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IN VITRO AND IN VIVO MEASUREMENT OF PERCUTANEOUS PENETRATION OF LOW MOLECULAR WEIGHT TOXINS OF MILITARY INTEREST

ANNUAL REPORT

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R.T. Riley; C.R. Clark

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The purpose of this contract is to: (i) use an in vitro system to assess the ability of low molecular weight toxins to penetrate through human and guinea pig skin, (ii) validate the results obtained in the in vitro system by conducting comparative studies of in vitro and in vivo cutaneous penetration in guinea pigs, (iii) assess the effects of specific solvent vehicles on the cutaneous penetration of the low molecular weight toxins, and (iv) modify the in vitro experimental system for skin penetration for use in studies of in vitro penetration across mucosal membranes. Toxins to be studied include the brevetoxins, microcystins, lyngbyatoxin A and debromoaplysiatoxin. Progress made during Year 2 of this contract is summarized below.

I. Effect of vehicles on the penetration of microcystin through excised mouse, guinea pig and human skin. Microcystin penetrated human skin but to a relatively small extent. The mean total penetration (expressed as percent dose, 100 ug) 48 hr after topical

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19. topical application was 0.9%, 2.2% and 3.8% when water, dimethylsulfoxide (DMSO), or methanol was the vehicle. Penetration of microcystin through human and guinea pig skin was not significantly different, however penetration through mouse skin was significantly larger. When the dose of microcystin applied to guinea pig skin was decreased, there was an increase in the percent of the dose which penetrated the skin.

II. Effect of vehicle on the penetration of [3H]PbTx-3 through excised human and guinea pig skin. [3H]Pbtx-3 penetrated through excised human skin but to a relatively small extent. The mean total penetration (expressed as percent dose, 0.3 ug) 48 hr after topical application to human skin was 0.9%, 3.1%, and 0.6% when water, DMSO, or methanol was the vehicle. The mean total penetration after topical application to excised guinea pig skin was 3.0%, 11%, and 4.5% when water, DMSO, or methanol was the vehicle, respectively.

III. Disposition of [3H]PbTx-3 after topical and subcutaneous application in weanling pigs. Total penetration of [3H]PbTx-3 through excised pig skin (expressed as percent of dose) during 24 hr was 0.3% when DMSO was the vehicle. Analysis of skin layers 2 hr after topically applying [3H]PbTx-3 to excised pig skin indicated the 7.2%, 2.8% and 0.5% of the dose had penetrated into the epidermis, dermis, and subcutaneous fat. These results indicate that PbTx-3 rapidly penetrates into the skin but then slowly diffuses into the receptor fluid which bathes the dermal surface in vitro. Subcutaneous application of [3H]PbTx-3 to pigs resulted in distribution of radioactivity to the urine (11%), feces (9%),muscle (12%), liver (3.5%), kidney (0.07%). and spleen (0.12%) by 3.2 days post application. Topical application of [3H]PbTx-3 to pigs resulted in excretion of radioactivity in the urine (0.5%) and feces (1%) by 4.2 days post application. In vivo skin absorption of [3H]PbTx-3 was calculated to be 8.5% (based on excretion method) and 23% (based on analysis of difference method). These results indicate that determining in vitro skin absorption by measuring only toxin in receptor fluid may underestimate skin absorption in vivo. It is important to also measure the amount of toxin which penetrates into the dermis and would be available for absorption in an in vivo situation.

IV. In vitro permeability of monkey buccal mucosa and skin to tritiated water (THO) and PbTx-3. The permeability of monkey buccal mucosa relative to skin was 16 and 9 times greater for THO and [3H]PbTx-3, respectively. The large amount of [3H]PbTx-3 which accumulated in the buccal mucosa and skin (46 and 24% of the dose, respectively) indicated that PbTx-3 readily penetrated into the skin, but slowly penetrated into the receptor fluid. In contrast, only 2 and 8% of the dose of THO was recovered within the buccal mucosa and skin (respectively) 24 hr after applying the dose.

V. Development of extraction procedure and chromatographic method to measure lyngbyatoxin A in skin extracts and aqueous receptor fluid. Solid phase extraction columns and liquid/liquid extraction has been used to try to develop an efficient and consistent method to extract lyngbyatoxin A from aqueous receptor fluid. The best method to date is liquid/liquid extraction using chloroform (69% of toxin was recovered).

19. VI. Evaluate viability of excised skin and buccal mucosa by histological examination and measurement of lactate dehydrogenase (LDH) release. Results of LDH release study were difficult to interpret and we need to repeat this study with a control group. Histological examination of excised buccal mucosa and skin indicated that these tissue appear normal after being incubated in the diffusion cells for 1 to 12 hr. When incubated for 24 and 48 hr there were minor histological changes which included pyknosis and swelling of the epithelial cells in the epidermis and hair follicles.

FOREWORD

a. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

b. In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication 86-23, Revised 1985).

c. For the protection of human subjects the investigators (s) have adhered to policies of applicable Federal Law 45CFR46.

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I. EFFECT OF VEHICLE ON PENETRATION OF MICROCYSTIN (A BLUE GREEN ALGAE TOXIN) THROUGH EXCISED MOUSE, GUINEA PIG AND HUMAN SKIN

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STATEMENT OF PROBLEM

It has been reported in various studies that microcystin poses a health risk to humans (Falconer et al. 1983). So far no study has been done to determine if skin exposure to water contaminated with microcystin would result in the penetration of microcystin through human skin.Due to extreme toxicity, it is not possible to conduct in vivo studies in humans. The purpose of this study was to determine if microcystin can penetrate excised human skin. The specific objectives were to determine: 1) the extent to which microcystin penetrates excised human skin, 2) if excised mouse and/or guinea pig skin provides a good model for human skin, 3) the effect of vehicle [water, dimethylsulfoxide (DMSO) and methanol] on penetration of microcystin through human, mouse and guinea pig skin and 4) to determine the effect of dose of microcystin on penetration through guinea pig skin.

BACKGROUND

Microcystin is a cyclic peptide (figure 1), produced by some strains of Microcystis aeruginosa (a blue green algae) (Krishnamurthy T., Szafraniec L., Sarver E. W. et al 1985; Krishnamurthy T., Carmichel W. W. and Sarver E. W. 1986). Toxic blooms of several strains of blue green algae (cynobacteria) are found worldwide in both natural and man made fresh water lakes. The most favorable conditions for a bloom to occur are warm, dry, low wind days of summer and early fall.Increased pollution in urban, recreational and agricultural water sources seems to contribute to the growth of toxic and nontoxic blooms (Skulberg, O.M., Codd, G. A. and Carmichael, W.W., 1984). Consumption of toxin in water and bloom mass have been implicated in the loss of live stock and wild animals in several countries throughout the world (Carmichael, W. W., Jones, C. L. A., Mahmood, N. A. and Theiss, W. 1985; Skulberg et al., 1984; Beasley, V.R., Coppock, R.W.,Simm,J., 1983), as well as human intoxication (Billings ,1982; Falconer, I.R., Beresford, A.M. and Runnegar, M.T.C.,1983). No known incident of lethal human poisonings by blue green algal toxins are reported. There is evidence that contact irritations and gastroenteritis occur when swimmers come into contact with toxic blue green algae cells or water containing the released toxin (Skulberg et al. 1984). It is not known if the toxin(s) were absorbed percutaneusly or via ingestion. There is increasing concern that toxic blooms might pose a health risk to people through 1) drinking contaminated water supplies and 2) recreational water.

Rats and mice injected with acutely toxic doses of M.aeruginosa cells or toxin extract die within 1 to 3 hr. Liver damage has been noted as early as 15 minutes and it has been suggested that liver damage is a direct effect of toxin on the hepatocyte membrane (Aune and Berg, 1986). The immediate cause of death in acutely dosed animals is hemorrhagic shock (Runnegar and Falconer 1982; Ostensvik,O., Skulberg, O.M. and Soli, N.E., 1981).

MATERIALS AND METHODS

MATERIALS

Microcystin was generously supplied by Dr. J.G.Pace, U.S. Army, Frederick, MD. Microcystin supplied was further purified by high pressure liquid chromatography (HPLC) to a purity of greater than 99%, see figure 2. HPLC was performed with a unit from Waters Associates (Milford, MA), which consisted of a U6K Injector, Model 510 Pump, Model 490 Variable Wavelength Detector, Model 740 Data Module and Model 712 Waters Intelligent Sample Processor (WISP). Reverse phase chromatography was used. Either Waters uBondapack C18 steel column (10 um, 3.9 mm x 36cm) or Hypersil ODS column (5 um, 50 x 4.6 mm, Keystone Scientific, Inc., State College, PA) was used. Eluant was 0.1 M ammonium acetate (pH 4 to 6) :acetonitrile (75:25, v/v) and flow rate was 0.5 ml/min. Absorbance was monitored at 240 nm. Microcystin peak had retention time of 8 to 10 min. To verify the identity of microcystin peak, eluant was collected from 8 to 10 min. with a fraction collector (ISCO Inc.,lincoln,Nebraska). Collected eluant was dried down under nitrogen stream and residue was dissolved in normal saline.

ICR mice weighing 27.4 + 0.5 gm (mean ± SD) were dosed i.p. with 20, 40 and 100 ug/kg of microcystin in 20, 40 and 100ul of normal saline, respectively. For the control group of mice, normal saline was injected on HPLC, eluant was collected at the same retention time as that of microcystin peak (8 - 10 min), evaporated to dryness and was dissolved in normal saline. No effect was observed in the control group. This was done to make sure that toxicity of HPLC eluant was not due to HPLC contaminant, but was due to microcystin eluting at 8 - 10 min. LD50 was determined to be 40 ug/kg, i.p. These results are consistant with the report by Eriksson, J.E., Meriluoto, J.A.O., Kujari, H.P. and Skulberg, O.M., 1988. The stability of microcystin in the receptor fluid was evaluated by mounting Teflon discs, instead of skin , on two diffusion cells in each group. The receptor fluid was dosed with microcystin (100 ug/25ul of DMSO, 100ug/50 ul of MEOH,or 5 ug/100 ul of water). The cells with Teflon discs were incubated along with cells with skin discs, in the environmental chamber (37°C). Each treatment group was replicated at least twice. Samples from receptor fluid were prepared and analyzed as described below.

Mouse skin was obtained from 13 female ICR mice weighing 30 ± 2 grams (mean + SD). Guinea pig skin was obtained from 4 male Hartley guinea pigs, weighing 550 ± 10 grams (mean + SD). Mice and guinea pigs were purchased from Harlan Sprague Dawley Inc., Indianapolis, IN. Animals were killed using carbon dioxide and ventral surfaces were carefully shaved with electric clippers before excising the skin. Human skin was obtained from autopsies

(performed within 24 hours of death) of 3 males (ages 47 to 79 years) and 2 females (ages 64 & 70 years). Loose subcutaneous fat was removed from excised skin. Split thickness human skin (thickness 500 - 650 um) was prepared with a Padgett dermatome, obtained from Padgett Dermatome, Division of Kansas City Assemblage Co.(Kansas city, MO). Full thickness mouse and guinea pig skin was used. The thickness of mouse skin was 600 to 700um and of guinea pig skin was 650 to 800 um.

METHODS:

Discs of excised skin, each 2.8cm2 in diameter, were mounted on static teflon diffusion cells (Riley, 1983), consisting of upper and lower chamber, see figure 3. The lower chamber (dermal side), has a volume of 2.6 ml and was filled with fluid (receptor fluid). Receptor fluid used was phosphate buffered saline (PBS) containing 157 mg penicillin/litre, 250 mg streptomycin/litre and 250 mg amphotericin B/litre to reduce bacterial or fungal growth. In some of the experiments, instead of PBS, Hanks Balanced Salt Solution (HBSS) with HEPES buffer and gentamycin 50 mg/litre was used as receptor fluid. HBSS was bubbled with 95% oxygen and 5% carbon dioxide to keep the skin discs viable (S. W. Collier, N M Shieth et al). The skin discs were placed horizontally between the two chambers, so that the dermal side was bathed by the receptor fluid and epidermal surface was exposed to ambient conditions in environmental chamber for 48 hr. Temperature and relative humidity during the length of experiment was 36 $\pm$3°C and 29 $\pm$ 5% , (mean $\pm$SD), respectively.

Skin from one specimen was mounted on at least one diffusion cell in each group (3 - 6 cells/group) so that variations in skin were evenly distributed in each group. Each experiment was repeated at least one time . At time 0, epidermal surfaces of mouse, guinea pig and human skin were dosed with 32- 36 ng/cm2 of microcystin dissolved in 50 ul of methanol or 25 ul of DMSO or 2 ml of water. Epidermal surfaces were not occluded when methanol or DMSO were vehicle but were occluded when water was vehicle. This was done to simulate a condition of a person swimming or walking in water contaminated with blue green algae toxin.

During all the experiments, some of the skin discs were dosed only with vehicle,i.e., with 2 ml of water, 25 ul of DMSO and 50 ul methanol.Cells were incubated under similar conditions along with other cells. At the end of experiments, receptor fluid and skin discs dosed with vehicle only were analyzed in a similar manner as the skin discs dosed with microcystin dissolved in these different vehicles. This was done to determine if there are any peaks due to components leaching out of skin due to the effect of vehicle only.

To determine the effect of dose on penetration through guinea pig skin, epidermal surfaces were dosed with 3.6, 7.2 and 32 μg/cm2 of microcystin dissolved in 10, 20 and 25 ul of DMSO.

At the end of each experiment (i.e., after 48 hr of incubation), the surfaces of some skin discs were washed with soap and water to determine the amount of dose remaining on skin surface . Each skin disc was then extracted twice with 10 ml of acetonitrile. This procedure involved homogenizing the skin with

a Brinkman homogenizer, centrifuging the homogenate and collecting the supernatant fluid which was then dried under a gentle stream of nitrogen. The residues were immediately dissolved in methanol. Receptor fluids from each experiment were prepared for injection on HPLC by using 3 ml C18 solid phase extraction columns (SPE, J.T. Baker, Phillispurg, NJ or Bond Elut, Analytichem International, Harbor City, CA).

Recovery of microcystin from SPE columns was determined to be 95 ± 3 percent of sample (mean ± SD). Each column was primed with methanol followed by PBS or HBSS. Receptor fluid was then aspirated and microcystin eluted with methanol or acetonitrile. Eluant was evaporated to dryness under nitrogen stream and dissolved in methanol for injection on HPLC.

Total recovery of the dose applied to each skin disc was calculated by summing the amount recovered from skin extracts, amount penetrated into receptor fluid and amount recovered from dose remaining on the epidermal surfaces.

Statistical analysis:

Percent of dose penetrated was analyzed by PC ANOVA (Human System Dynamics, Northridge, California), and significant mean differences were estimated by Newman-Keuls test.

RESULTS AND DISCUSSION

Penetration through human skin and effect of vehicle

Microcystin does penetrate the human skin, but to a relatively

small extent. Over a period of 48 hr, only 0.9±0.3%, 2.2±0.8% and 3.8±0.06% (mean ± SD) of total dose applied penetrated through human skin when water, DMSO or MEOH were vehicle, respectively (Table 1). Microcystin penetration through human skin was not significantly different when water or DMSO was the vehicle; penetration was significantly faster when methanol was the vehicle ($P < 0.05$). At the end of the experiment, 12% of dose was in the skin when water or DMSO was the vehicle, 73% and 60% was left on the epidermal surface when water or DMSO was the vehicle,respectively. Total dose recovered at the end of the experiment was larger when water was the vehicle (92%) than when methanol or DMSO was the vehicle (73 and 76%, respectively). The relatively low recovery of total dose when methanol or DMSO was the vehicle, could be due to difficulty in extracting the microcystin from skin. Dose left over epidermal surface also was larger when water was the vehicle (73%) than when methanol or DMSO were the vehicles (68 and 60%, respectively). Microcystin which penetrated into the skin may ultimately penetrate through skin over a course of time. DMSO is a dipolar aprotic solvent and is thought to displace bound water from skin which results in a looser structure (Scheuplein and Bronaugh, 1983). The penetrant is thought to mainly diffuse through the DMSO in the membrane. DMSO may alter the skin on contact, diffuse rapidly through it, increase its permeability, and thereby promote the penetration of materials dissolved in them (Allenby, A.C., Crasey, N.H., Edginton, J.A.G., & Shock, C. 1969). However, the structural alteration of epidermis produced by DMSO and its analogues is to

some degree reversible (Baker, H. 1968). The rationale for use of DMSO was to: 1) enhance movement of toxin across the membrane of skin (Wood and Wood, 1975), which can simulate the effects of abrasion on dermal penetrant, 2) to determine the extent of penetration if personnel working in laboratory, who are involved in extracting microcystin, accidentally come in contact with microcystin in the presence of penetration enhancer .

HPLC analysis of receptor fluid and skin extract from skin discs dosed with the vehicle only, showed only solvent peaks. This indicates that if constituents of skin leached out of the skin into the receptor fluid, then these constituents either had different retention times or were not strong UV (ultraviolet) absorbers, figure 4.

HPLC analysis of receptor fluid and skin extacts from the human skin discs dosed with microcystin dissolved in different vehicles showed no peaks in addition to solvent peaks and microcystin peak. Samples prepared from mouse and guinea pig skin dosed with microcystin in DMSO also did not show any peaks in addition to microcystin and solvent peaks. These results are consistent with the hypothesis that no cutaneous metabolism of microcystin occured in human, mouse and guinea pig skin when DMSO was the vehicle, or in human skin when water and methanol were used as vehicles, figure 5.

Penetration through different species

Penetration through mouse skin was significantly larger ($P < 0.05$) than through human skin (figure 6). Penetration through guinea pig skin was greater than through human skin, but was not

statistically significantly different. Penetration through human, mouse and guinea pig skin was compared when DMSO was the vehicle. These results infer guinea pig skin to be a good model of microcystin penetration through human skin when DMSO is the vehicle. These results are consistent with literature that the guinea pig is a reasonable model for human skin absorbtion

Effect of different doses of microcystin on penetration

Effect of different doses of microcystin on penetration was studied with guinea pig skin. Guinea pig was chosen for this purpose, because this species was determined to be a good model of microcystin penetration through human skin. Percent of dose penetrated decreased as the dose was increased, refer to figure 7. Vehicle for the dose applied was DMSO. Percent of dose penetrated was not significantly different when microcystin dose was increased from 8.5 ug to 17 ug but was significantly different when dose was increased to 88ug ($P < 0.05$). The decrease in percent penetration observed at high doses could be due to saturation of the absorption process (Wester and Maibach, 1983). Increased dose decreased the percent of dose penetrated, but total amount of dose penetrated was increased. This could be due to the effect of DMSO which acts as a penetration enhancer.

CONCLUSION

In conclusion, guinea pig skin is a good model of microcystin penetration through human skin. The results of this study indicate microcystin penetrates excised skin relatively slowly if the

vehicle is water, DMSO or methanol. These findings suggest that short term cutaneous exposure to microcystin in a laboratory in the presence of a solvent or through recreational water would not pose a health risk .

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Table 1. Effect of vehicle on penetration of microcystin through human skin[a].

Vehicle (Sample Size)	Amount Penetrated	Amount in Skin	Amount left over epidermal surface	Total Recovery
Water (6)	0.9 ± 0.3[b]	12 ± 3.9	72.9 ± 8.8	92.1 ± 7.1
DMSO (10)	2.2 ± 0.8[b]	12 ± 3.6	59.8 ± 6.9	75.8 ± 7.4
Methanol (4)	3.8 ± 0.06[c]	----	67.6 ± 4.5[d]	72.5 ± 4.3

[a]Values represent distribution of microcystin 48 hr after applying the dose on skin. Values are expressed as PERCENT DOSE (mean ± standard deviation).

[b,c]Values with different superscripts are significantly different ($P \leq 0.05$).

[d]Includes amount in skin and left on epidermal surface.

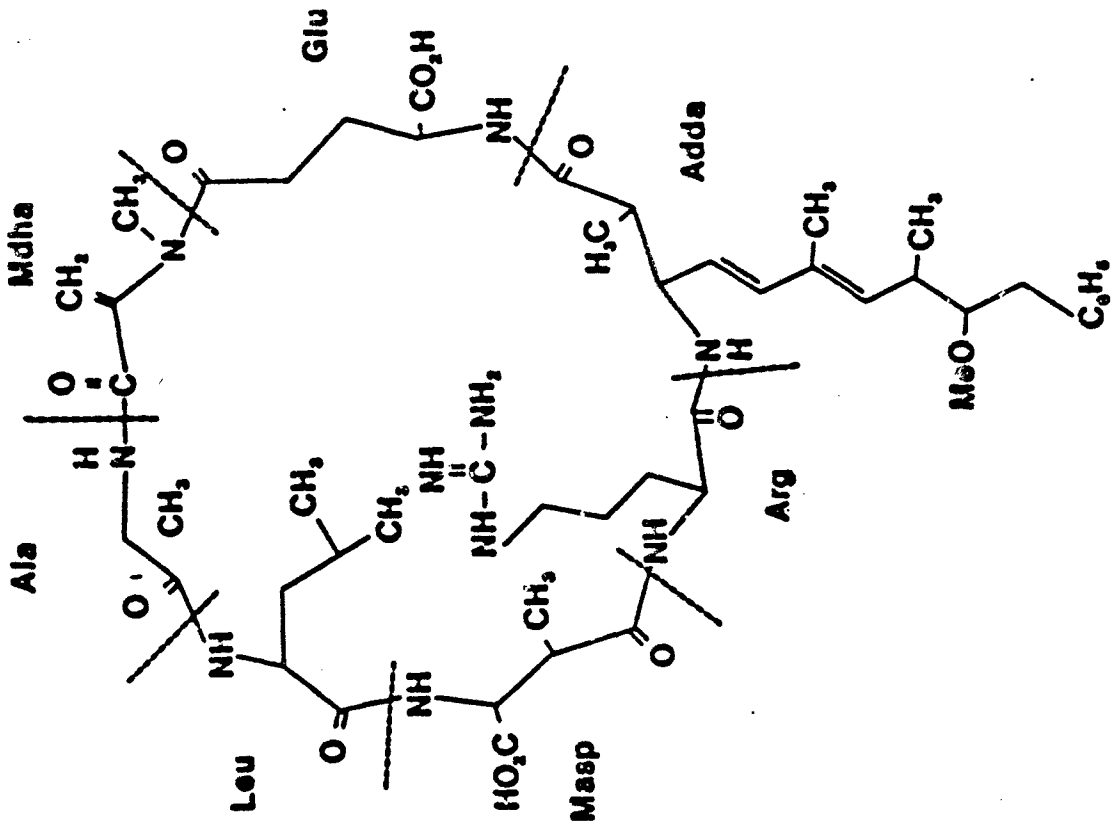

Figure 1. Structure of microcystin.

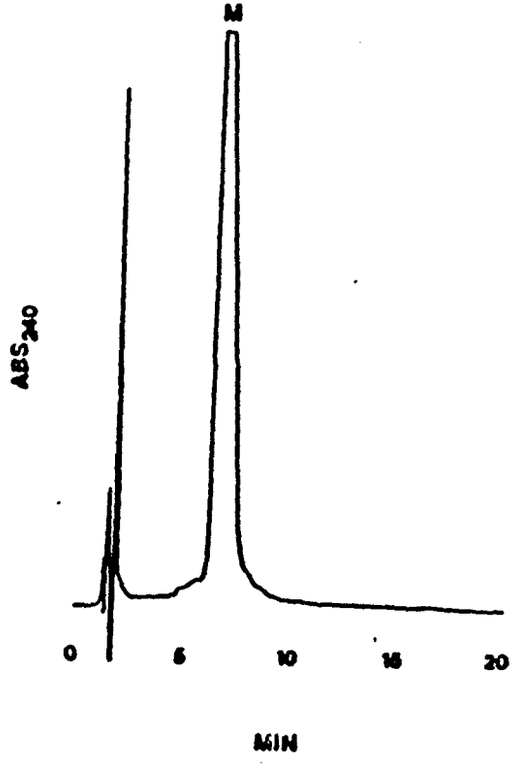

Figure 2. High performance liquid chromatography of purified microcystin (2.5 ug), M = microcystin peak. Chromatographic conditions were: reverse phase C18 uBondapak column, 10um, 3.9 mm x 36cm; mobile phase - 0.1 M ammonium acetate (pH 4-6) : acetonitrile (75:25, v/v); flow rate - 0.5 ml/min; wavelength was monitored at 240 nm.

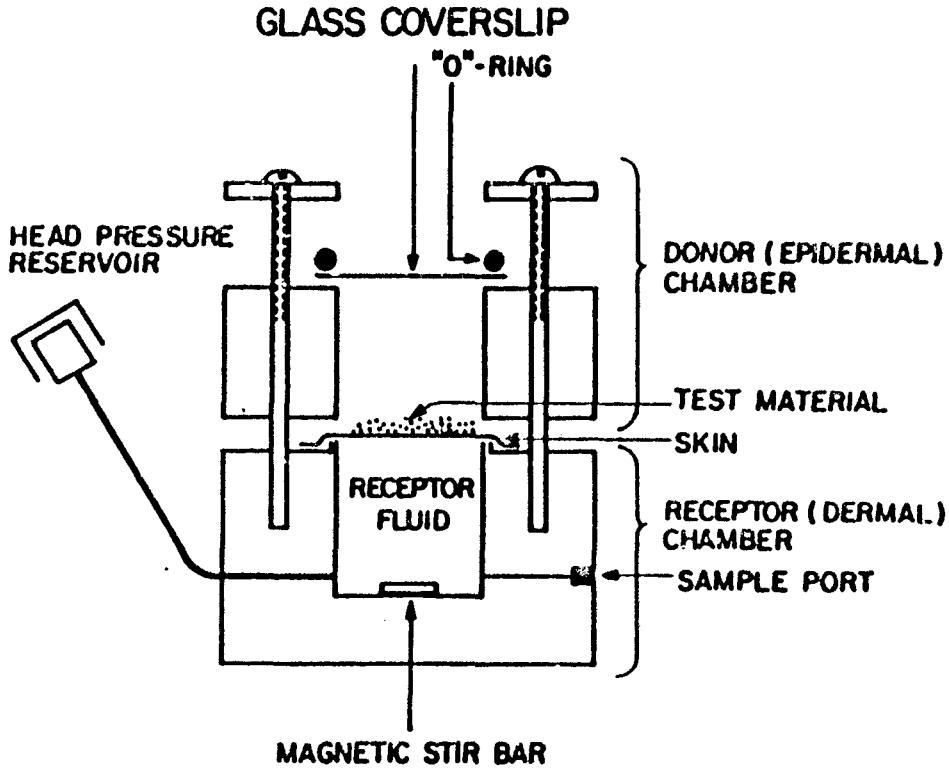

Figure 3. Static diffusion cell used for skin penetration studies.

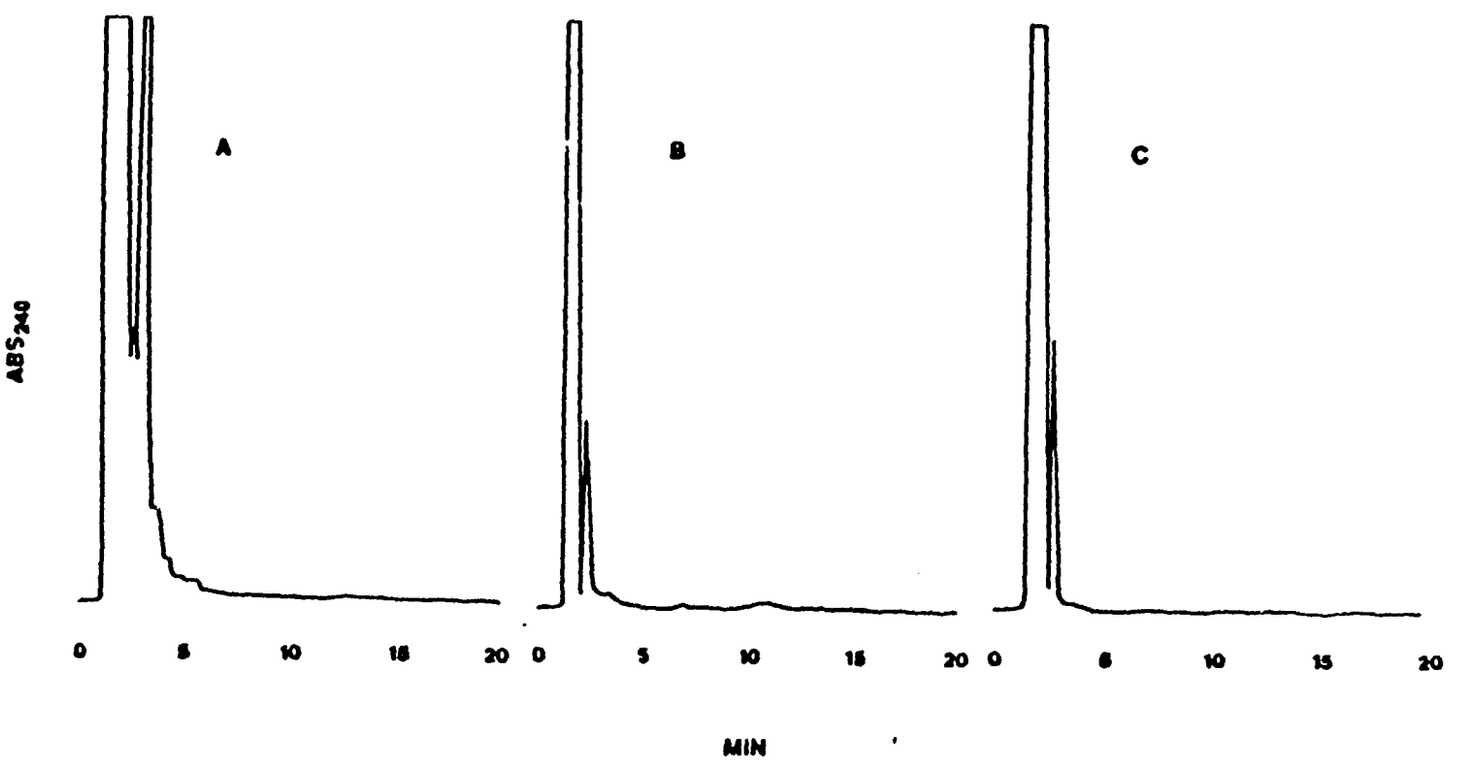

Figure 4. HPLC chromatographic analysis of receptor fluid from human skin dosed only with vehicle, A. 2 ml of water, B. 25ul of DMSO and C. 50 ul of methanol. Chromatographic conditions were same as described in figure 2.

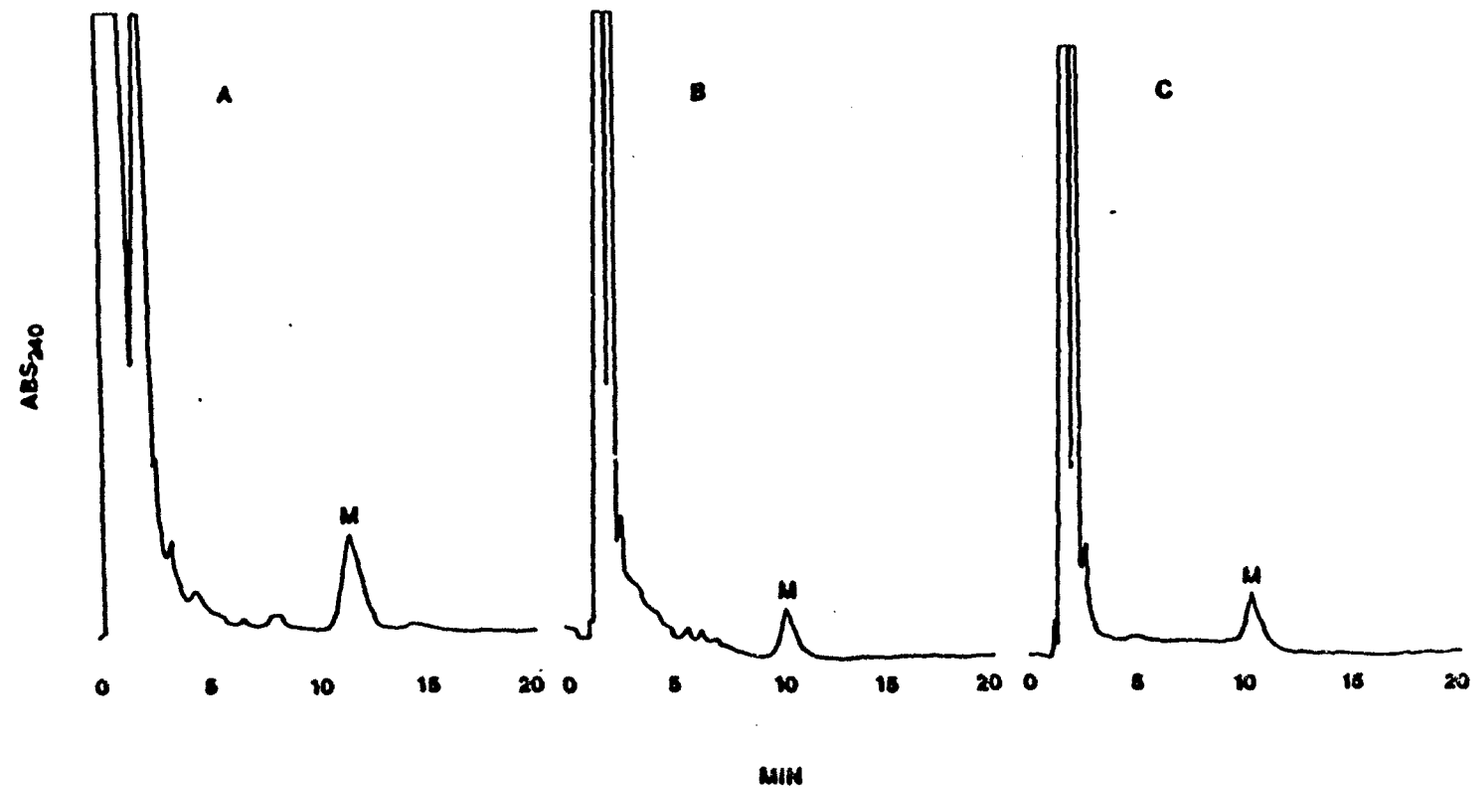

Figure 5. HPLC chromatographic analysis of receptor fluid from human skin dosed with microcystin dissolved in, A. 2 ml of water, B. 25 ul of DMSO and C. 50 ul of methanol. M = microcystin. Chromatographic conditions were same as described in figure 2.

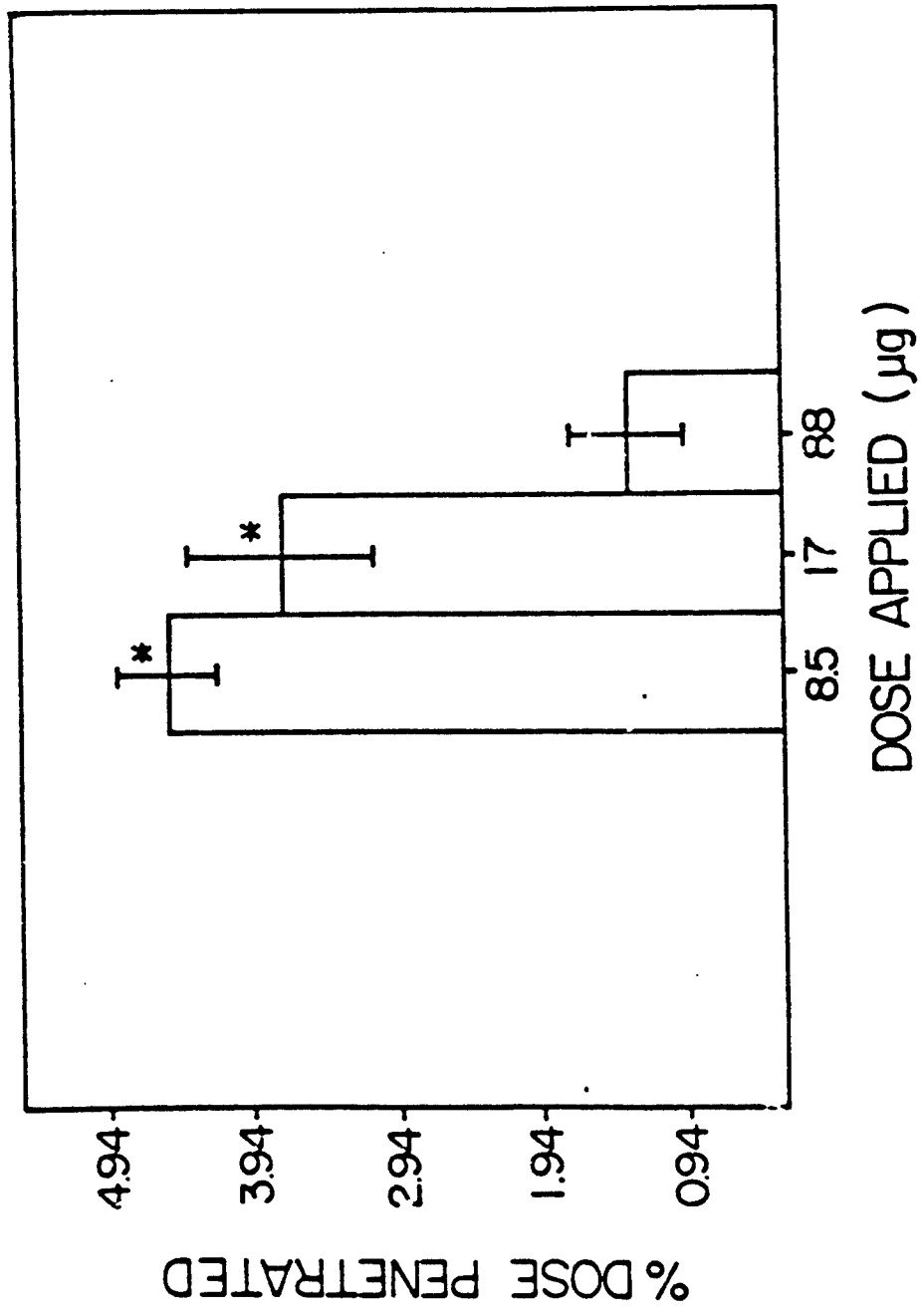

Figure 6. Penetration of microcystin through human, guinea pig and mouse skin (vehicle = DMSO). Asterisks indicate values not significantly different from each other ($P > 0.05$).

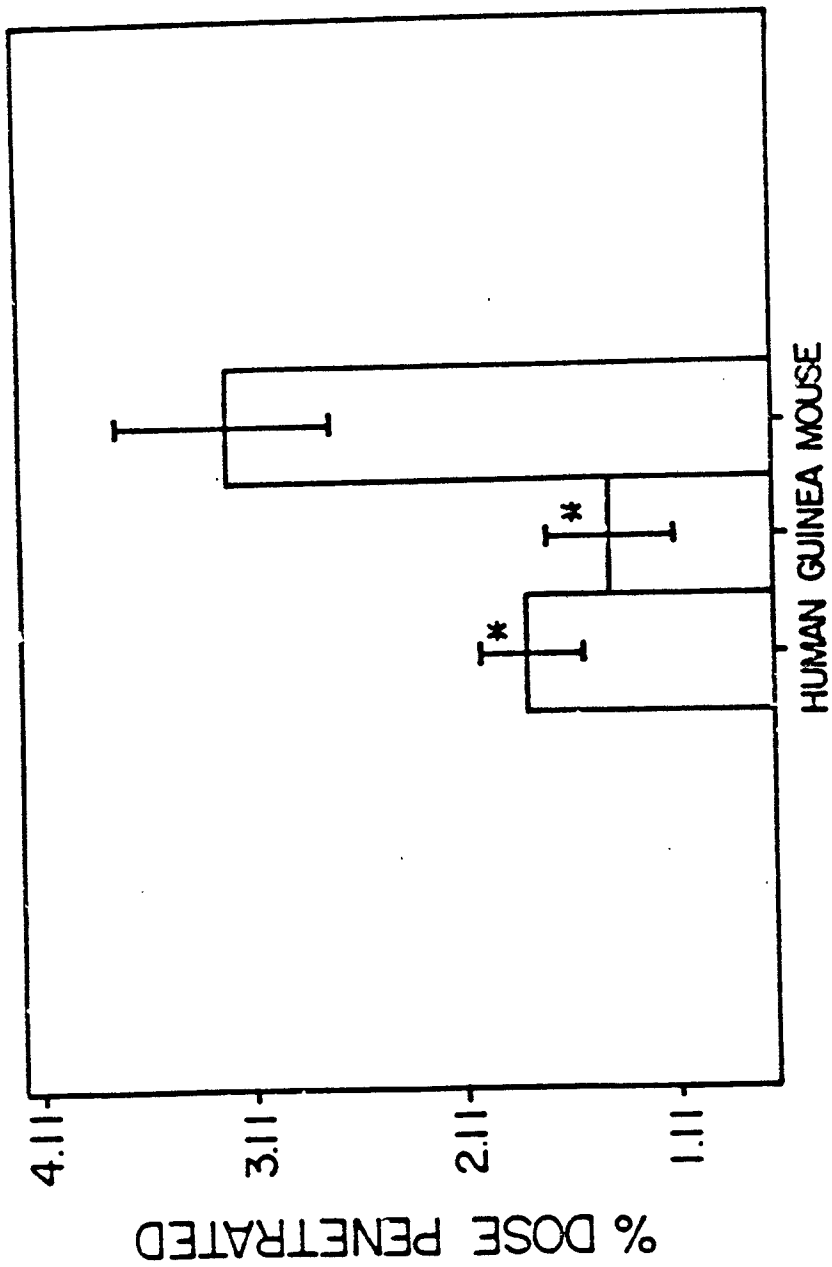

Figure 7. Effect of dose on penetration of microcystin through guinea pig skin. Asterisks indicate values not significantly different from each other ($P > 0.05$).

II. EFFECT OF VEHICLE ON THE PENETRATION OF [3H]PbTx-3 (A RED TIDE TOXIN) THROUGH EXCISED HUMAN AND GUINEA PIG SKIN

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STATEMENT OF PROBLEM

The purpose of this research was to determine the amount of absorption which would occur if humans were cutaneously exposed to [3H]PbTx-3. The specific objectives were to (i) compare the penetration of [3H]PbTx-3 through excised human skin using static and flowing diffusion cells, (ii) use flowing diffusion cells to compare the penetration and metabolism of [3H]PbTx-3 in excised human cadaver abdominal skin and excised human surgical breast skin when methanol and dimethylsulfoxide (DMSO) are the vehicles, (iii) determine the effect of vehicle (methanol, water, and DMSO) on the penetration of [3H]PbTx-3 through human skin, and (iv) compare penetration of [3H]PbTx-3 through excised human and guinea pig skin.

BACKGROUND

PbTx-3 is one of a group of brevetoxins produced by the marine dinoflagellate P. brevis. This algae has a soft outer covering which ruptures in the surf and results in the release of toxins. Blooms of P. brevis are referred to as red tide. People and animals walking along the seashore during a red tide experience coughing and bronchoconstriction. People swimming in contaminated water experience skin irritation in the form of redness and itching. It is not known if brevetoxins can penetrate skin and cause systemic effects. People are also exposed to brevetoxins in laboratories when culturing and extracting these toxins for use in research. During the extraction procedures the toxins may be in high concentrations and combined with solvents which could enhance their rate of skin penetration. Therefore, it is important to determine the rate of skin penetration of brevetoxins in the presence of several different vehicles.

RATIONALE USED IN CURRENT STUDY

PbTx-3 is extremely toxic. The LD50 in mice is 0.2 mg/kg via the intravenous or intraperitoneal route of administration (Baden and Mende, 1982). Therefore it is unethical to study the skin absorption of PbTx-3 in living human subjects. An in vitro method has been developed for measuring the penetration of compounds through excised skin. The main barrier to diffusion of xenobiotics through skin is the stratum corneum. Since the stratum corneum is

comprised of dead keratinized cells it maintains its barrier properties when skin is excised. The validity of using excised skin to determine skin penetration of xenobiotics was tested by comparing the *in vitro* and *in vivo* skin penetration of several compounds (see Table 1). In Table 1 the penetration of compounds through excised skin using static and flowing diffusion cells is compared to *in vivo* skin absorption. In the static system, penetration was measured by removing sequential samples from the stirred solution (receptor fluid) which bathes the dermal surface of the excised skin disc. In the flowing system, receptor fluid was continuously pumped through the chamber below the skin. In 6 of the 8 comparisons, *in vitro* and *in vivo* skin penetration differed by factors of between 1.1 and 1.6 (average difference was a factor of 1.3). It was concluded that in most cases there was good correlation between *in vitro* and *in vivo* skin penetration. The exceptions were: (i) *in vitro* penetration of urea in the flowing cell overestimated *in vivo* penetration by a factor of 6 and (ii) *in vitro* penetration of T-2 toxin (when methanol was the vehicle) using the static cell underestimated *in vivo* absorption by a factor of 6. The latter exception is probably an example of decreased correlation between *in vitro/in vivo* skin penetration for lipophilic compounds due to low solubility in aqueous receptor fluid. This is usually not a problem when DMSO is the vehicle for a lipophilic compound, because DMSO readily penetrates the skin and acts as a solubilizing agent in the receptor fluid (see Table 1).

In the following study both static and flowing diffusion cells were

used to determine if penetration of PbTx-3 through excised human skin was artificially reduced due to limited solubility in the aqueous receptor fluid. It was hypothesized that since the volume of receptor fluid in the flowing cell (4 ml/hr x 48 hr = 132 ml) was considerably larger than the volume of receptor fluid in the static cell (2.5 ml), if solubility was a limiting factor than penetration would be greater in the flowing cells than in the static cells.

It has been suggested (Bronaugh, personal communication) that the metabolic activity of human surgical skin samples is more intact than that of human cadaver skin samples. Logically, this would appear to be true, but we are unaware of any data to support this hypothesis. The penetration and metabolism of [3H]PbTx-3 was compared in excised human cadaver abdominal skin and excised surgical breast skin in order to determine if metabolism of PbTx-3 was different in these 2 tissues.

Studies comparing the toxicity and absorption of algal toxins (including brevetoxins) following cutaneous application to guinea pigs have been done at Fort Detrick (Wannemacher, Bunner, and Dinterman, 1988). In the following study the penetration of [3H]PbTx-3 through excised guinea pig and human skin has been compared in order to determine if guinea pig skin is a good model of skin absorption of PbTx-3 in humans.

EXPERIMENTAL METHODS

Materials: [3H]PbTx-3 was purchased from Daniel Baden, Miami, FL and had a specific activity of 10 to 13 Ci/mmol. PbTx-3 was also purchased from Baden and used as a standard for chromatographic analysis. High performance liquid chromatography (HPLC) was used to determine the purity of the [3H]PbTx-3. The HPLC methods involved using C18 reverse phase uBondapak column (Waters, Milford, MA), mobile phase of methanol:water (85:15), flow rate of 0.5 ml/min, and monitoring radioactivity with a FLO-ONE (Packard Instruments, Tampa, FL). The purity of the [3H]PbTx-3 was determined to be greater than 98%.

Human cadaver and abdominal skin was obtained from a hospital pathology department. The skin was placed in air-tight plastic bags, boxed with wet ice and shipped via overnight delivery within 24 hr of death/surgery. Split-thickness human skin (500 um) containing the epidermis and part of the dermis was prepared by using an electric dermatome (Padgett, Kansas City, MO). Male Hartley guinea pigs were purchased from Hilltop Lab Animals (Scottdale, PA). Guinea pigs were killed immediately prior to an experiment with CO2, abdominal surface clipped with electric clippers, and full thickness skin excised (600 to 800 um).

In vitro skin penetration experiments: Human and guinea pig skin discs (each with a surface area of 2.8 cm2) were mounted on static teflon diffusion cells. The dermal surfaces were bathed by 2.5 ml

receptor fluid (phosphate buffered saline with antibiotics, PBSA; or Hanks Balanced Salt Solution with HEPES buffer and gentamicin, HBSS). The receptor fluids were gassed with O2:CO2 (95:5) prior to the experiment. The dose (301 to 334 ng [3H]PbTx-3/cm2) was dissolved in methanol (50 ul), DMSO (60 ul), or water (2000 ul) and applied to the epidermal surface of the skin disc. The epidermal surfaces of the skin discs dosed with [3H]PbTx-3 dissolved in methanol or DMSO were not occluded in order to simulate a condition were a person in a laboratory had toxin dissolved in a solvent spill on them and then it was allowed to air dry. The surfaces dosed with [3H]PbTx-3 dissolved in water were occluded to reduce evaporation and simulate a condition were a person was walking or swimming in water contaminated with brevetoxin. The diffusion cells were incubated in an environmental chamber (37 degrees C) for 48 hr and sequential samples (50 ul) were removed from the receptor fluid. Radiochemical composition of the receptor fluid was determined after 24 and 48 hr by injecting an aliquot (10 to 50 ul) of receptor fluid on the HPLC/FLO-ONE. At the end of each experiment the epidermal surfaces were washed with soap and water and Q-tips in order to determine how much of the dose remained on the skin surface. The Q-tips were then extracted with 10 ml methanol. The skin discs were each extracted twice with 10 ml of methanol. The amount of radioactivity in the extracts of the Q-tips and skin was individually determined with liquid scintillation (LSC) techniques. The radiochemical composition of the skin washes and skin extracts was determined by injecting an aliquot of each sample on the HPLC/FLO-ONE. The total recovery of the dose was

determined by summing radioactivity in receptor fluid, skin decontamination, and skin extracts.

For the skin penetration studies using flowing diffusion cells, skin discs with a surface area of 0.79 cm2 were mounted on glass flowing diffusion cells. These diffusion cells have a receptor volume of 3.0 ml. The receptor fluid was continually replaced by pumping receptor fluid (HBSS) through the cells at rate of 4 ml/hr with a peristaltic pump. A fraction collector (Gilson Medical Electronics, Middleton, WI) was used to collect 4 hr samples of the receptor fluid which contained [3H]PbTx-3 and metabolites which had penetrated through the skin. Radioactivity in the receptor fluid, on the skin surface, and within the skin was determined as described above. In order to determine the radiochemical composition of the receptor fluid it was necessary to first pool samples and extract them using C18 solid phase extraction columns (Bond Elut, Analytichem International).

SAS General Linear and General Curvilinear Models were used to compare: (i) the effects of diffusion cell design, (ii) different types of skin, and (iii) the effects of different vehicles on the penetration kinetics of [3H]PbTx-3 through excised skin. For each group the observations from all the experiments were fitted to linear, quadratic, and cubic functions of time (i.e., vel = a + b1hr + b2hr2 etc). The model which best fit the data was determined based on the "t" statistic for the parameters hr, hr2, and hr3. The least squares regression model which best fit the data

was then segmented into linear components by a process of adding or deleting the observations at the times which proceeded or followed the point at which visually estimated linear segments intersected. When the first order model (vel = a + b1hr) provided the best fit of the data then these times ("hr"), slopes ("change in velocity", and predicted values for the maximal velocity ("max vel") were taken to define that linear segment. When the least squares regression analysis indicated that there was no relationship between hr and velocity ($P > 0.05$), the slope was given a value of 0.

RESULTS AND DISCUSSION

The penetration of [3H]PbTx-3 through excised human skin using static and flowing diffusion cells is compared in Table 2. The kinetics of penetration was markedly different in the static versus the flowing cell, however the total penetration after 48 hr was similar for the 2 cell designs (differed by a factor of 1.3). In the static cell the rate of penetration rapidly increased during 2 hr to 4 hr, followed by a rapid decrease in the rate of penetration during 16 hr to 33 hr. In the flowing cells the rate of penetration gradually increased during 4 hr to 24 hr and then the rate of penetration remained constant during 24 hr to 48 hr (110 pg/cm2/hr). The similar total penetration (after 48 hr) of [3H]PbTx-3 in the 2 cell designs suggests that limited solubility in the aqueous receptor fluid was not a problem. If solubility of [3H]PbTx-3 in the receptor fluid was a limiting factor then

penetration in the flowing cells would have been expected to be greater than in the static cells because of the large difference in volume of receptor fluid in the static cell (2.5 ml) compared to the flowing cell (132 ml).

The penetration of [3H]PbTx-3 through excised human cadaver abdominal skin and human surgical breast skin is compared in Table 3. Although the penetration is larger in the surgical breast skin than the cadaver abdominal skin whether the vehicle is methanol or DMSO, the kinetics of penetration are similar in these 2 types of skin. That is, when methanol is the vehicle the rate of penetration during the entire exposure period is characterized by a single linear segment and it has no slope (surgical) or very small slope (cadaver). When DMSO is the vehicle the rate of penetration is characterized by an increase during 4 hr to 24 hr followed by a decreased (negative slope) rate in the surgical sample or constant (no slope) rate in the cadaver sample during 24 hr to 48 hr.

The effect of vehicle on the penetration of [3H]PbTx-3 through excised human and guinea pig skin is shown in Tables 4 and 5, respectively. In both species penetration was largest when DMSO was the vehicle. Penetration through excised guinea pig skin was larger than through excised human skin by factors ranging between 3 and 8.

The recovery of the applied dose in the flowing cell experiments

is shown in Table 6. When methanol was the vehicle between 13 and 14% of the dose was within the skin, however when DMSO was the vehicle 30 to 52% of the dose was extracted from the skin. This is consistent with previous reports that DMSO acts to create a reservoir of the penetrant within the skin.

The stability of [3H]PbTx-3 in receptor fluid and vehicles is shown in Table 7. This radiolabeled compound was stable except that it broke down in aqueous solutions at the rate of approximately 10% per 24 hr, at 37 degrees C. The results of radiochromatographic analysis of the components of the flowing diffusion cells is shown in Table 8. There was some breakdown of the compound in the washes from the skin surface. This is thought to be due to chemical interaction with the soap used to wash the skin surface. There was some breakdown in the receptor fluid. This is thought to be due to instability of the radiolabel in aqueous receptor fluid. Typical chromatograms are shown in Figure 1.

CONCLUSION

[3H]PbTx-3 penetrates excised skin but at a relatively slow rate regardless of the vehicle. These results indicate that short term skin exposure to PbTx-3 would pose minimal risk of systemic toxicity.

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2. Wannemacher, R.W., Jr., Bunner, D.L., and Dinterman, R.E. (1988) Transdermal toxicity of several natural toxins from marine and aquatic environments. Presented:International Society on Toxinology Annual Meeting, Stillwater, OK, July 31 - August 5, 1988.

Table 1. Test validity of *in vitro* methods by comparing *in vitro* and *in vivo* skin penetration.

Penetrant	Species	Vehicle	Percutaneous Penetration Method[a]		
			in vivo	static in vitro	flowing in vitro
[^{14}C]Urea	Human	Acetone	6[b]	8[c]	36[c]
[^{14}C]Benzoic Acid	Human	Acetone	43[b]	48[c]	62[c]
[^{3}H]T-2	Guinea Pig	Methanol	23[d]	4	15
[^{3}H]T-2	Guinea Pig	DMSO	52	38	43

[a]Penetration expressed as mean percent dose of applied.

[b]Values from Feldmann and Maibach, 1970.

[c]Values from Riley, Kemppainen and Norred, 1985.

[d]Values from Kemppainen, Pace and Riley, 1987.

Table 2. Comparison of the kinetics of penetration of [^{3}H]PbTx-3 through excised human cadaver abdominal skin using static and flowing diffusion cells (vehicle was DMSO).

Diffusion Cell design	Component of model	Linear Segmentation of Least Squares Regression Model[d]			Penetration at 48 hr (%dose)[f]
		Segment 1	Segment 2	Segment 3	
Static	Time (hr)[a]	4 to 6	16 to 33	33 to 48	3.1 ± 2.0%
	Slope of Seg.[b]	40 ± 13	-28 ± 9	0	
	Max. Vel.[c]	532 ± 103	634 ± 102	164 ± 39	
	n	18	25	22	
Flowing	Time	4 to 24	24 to 48	NA[e]	2.4 ± 0.6%
	Slope of Seg.	6 ± 3	0		
	Max. Vel.	165 ± 31	110 ± 64		
	n	22	35		

[a]Time (hr) indicates the times which define each linear segment in hours.

[b]Slope of Segment indicates the rate of change in the velocity of penetration ($pg/cm^2/hr/hr$) which is the slope of the identified segment plus or minus standard error; a negative value indicates a negative slope.

[c]Maximum Velocity of penetration for the segment; where there was a significant slope the predicated value from the linear model at the time of maximal penetration plus or minus the standard error, is defined as the "max. vel.". When the slope was 0, the mean of all observations, plus or minus the standard error, for that time segment was taken as the "max. vel.".

[d]See Experimental Methods section for description of how Least Squares Regression Model was used to subdivide the penetration rates into linear segments.

[e]NA = Not Applicable.

[f]Percent penetration expressed as Mean ± standard deviation.

Table 3. Comparison of the kinetics of penetration of PbTx-3 through excised human cadaver abdominal skin and human surgical breast skin when methanol or DMSO were the vehicles. Flowing cells were used in these experiments.

Skin Source	Vehicle	Component of model	Linear Segmentation of Least Square Regression Model[a]		Penetration at 48 hr (% dose)[e]
			Segment 1	Segment 2	
Human Cadaver Abdominal	Methanol	Time[a]	4 to 48	NA	0.03 ± 0.02%
		Slope of Seq.[b]	0.03 ± 0.01		
		Max. Vel.[c]	2.5 ± 0.1		
		n	60		
	DMSO	Time	4 to 24	24 to 48	2.4 ± 0.6%
		Slope of Seq.	6 ± 3	0	
		Max. Vel.	165 ± 31	110 ± 64	
		n	22	35	
Human Surgical Breast	Methanol	Time	4 to 48	NA	0.09 ± 0.03%
		Slope of Seq.	0		
		Max. Vel.	4.6 ± 0.4		
		n	36		
	DMSO	Time	4 to 24	24 to 48	5.7 ± 2.5%
		Slope of Seq.	34 ± 7	-17 ± 5	
		Max. Vel.	814 ± 83	738 ± 87	
		n	54	65	

[a,b,c,d]See Table 1 footnotes for description of components of model.

[e]Percent penetration expressed as mean ± standard deviation.

Table 4. Effect of vehicle on the kinetics of penetration of [^{3}H]PbTx-3 through excised human cadaver abdominal skin.

Vehicle	Component of model	Linear Segmentation of Least Squares Regression Model[d]			Penetration at 48 hr (%dose)
		Segment 1	Segment 2	Segment 3	
Methanol	Time[a]	4 to 48	NA	NA	0.61 ± 0.66%
	Slope of Seg.[b]	2.1 ± 0.7			
	Max. Vel.[c]	95 ± 17			
	n	46			
DMSO	Time	4 to 16	16 to 33	33 to 48	3.1 ± 2.0%
	Slope of Seg.	40 ± 13	-28 ± 9	0	
	Max. Vel.	532 ± 103	634 ± 102	164 ± 39	
	n	18	25	22	
Water	Time	6 to 48	NA	NA	0.94 ± 0.57%
	Slope of Seg.	4.3 ± 1.3			
	Max. Vel.	157 ± 31			
	n	30			

[a,b,c,d]See Table 1 footnotes for description of components of model.

Table 5. Effect of vehicle on the kinetics of penetration of [^{3}H]PbTx-3 through excised guinea pig abdominal skin.

Vehicle	Component of model	Linear Segmentation of Least Squares Regression Model[d]			Penetration at 48 hr (%dose)
		Segment 1	Segment 2	Segment 3	
Methanol	Time[a]	5 to 48	NA	NA	4.9 ± 4.7%
	Slope of Seg.[b]	0			
	Max. Vel.[c]	326 ± 45			
	n	82			
DMSO	Time	6 to 18	18 to 48	NA	11 ± 3.3%
	Slope of Seg.	94 ± 15	-41 ± 6		
	Max. Vel.	1653 ± 115	1276 ± 130		
	n	16	36		
Water	Time	5 to 40	40 to 48	NA	3.0 ± 1.8%
	Slope of Seg.	10 ± 2	0		
	Max. Vel.	386 ± 42	340 ± 55		
	n	45	18		

[a,b,c,d]See Table 1 footnotes for description of components of model.

Table 6 . Total Penetration of [^{3}H]PbTx-3 Through Excised Human Skin and Recovery of Dose 48 hr After Dosing Skin Surface

Skin Source	Vehicle[a]	Recovery of PbTx-3 (expressed as % dose) Receptor fluid	Resident in skin	Skin Surface	Total Recovered
Human Cadaver Abdominal	DMSO	2.4±0.6[b] (9)	30±8.2 (11)	49±15 (11)	81±18 (9)
Human Surgical Breast	DMSO	5.7±2.5 (9)	52±17 (9)	25±14 (9)	83±5.9 (9)
Human Cadaver Abdominal	MEOH	0.03±0.02 (3)	14±6.2 (6)	70±18 (6)	84±13 (3)
Human Surgical Breast	MEOH	0.09±0.03 (3)	13±5.7 (3)	85±1.4 (3)	98±2.7 (3)

[a] Dose: 267 - 470 Nanograms [^{3}H]PbTx-3/ 37ul vehicle

[b] Expressed as Mean ± S.D. (n)

Table 7. Stability of [^{3}H]PbTx-3 in receptor fluid and vehicles used for penetration studies.

Substance analyzed	Chromatographic peaks[a]	
	Unknown R_T = 1.3 - 4.5	PbTx-3 4.2 - 9.3
Stock solution[b]	2	98
Solution in PBSA[c]	18	81
Solution in methanol[d]	1	99
Solution in DMSO[d]	4	96
Solution in water[d]	21	79

[a]See Experimental Methods section for HPLC methods used. R_T is the retention time in minutes. Values are expressed as mean percent of total peak area.

[b]Stock solution consisted of [^{3}H]PbTx-3 dissolved in methanol: water (85:15), which was stored at -20°C.

[c]Stability of [^{3}H]PbTx-3 in receptor fluid (phosphate buffered saline with antibiotics, PBSA) was determined by incubating (37°C) [^{3}H]PbTx-3 with PBSA for 48 hr.

[d]Stability of [^{3}H]PbTx-3 in each vehicle (methanol, DMSO, water) was determined by incubating (37°C) [^{3}H]PbTx-3 with each vehicle for 48 hr.

Table 8. Radiochromatographic Analysis of Components of Flow Diffusion Cells

Source of Skin	Vehicle	Component of Diffusion Cell	Chromatographic peaks[a]	
			Unknown R_t: 1.0-2.9[b]	PbTx-3 R_t: 3.5-6.2
Human Abdominal Cadaver	DMSO	Receptor Fluid	21±13[c] (23)	79±18 (23)
		Within Skin	2.3±6.9 (10)	98±7.0 (10)
		Skin Surface	58±4.4 (6)	40±3.1 (6)
Human Breast Surgical	DMSO	Receptor Fluid	28±10 (7)	68±18 (7)
		Within Skin	1.1±1.4 (10)	99±1.6 (10)
		Skin Surface	1.7±1.7 (4)	98±1.7 (4)
Human Abdominal Cadaver	MEOH	Receptor Fluid	_[d]	_[d]
		Within Skin	0 (6)	99±1.5 (6)
		Skin Surface	_[e]	_[e]
Human Breast Surgical	MEOH	Receptor Fluid	_[d]	_[d]
		Within Skin	0 (3)	100±0 (3)
		Skin Surface	_[e]	_[e]

[a] Expressed as % total peak area

[b] R_t = retention time (minutes)

[c] Expressed as Mean ± Standard Deviation (n)

[d] Not enough radioactivity to measure with HPLC

[e] Data not available

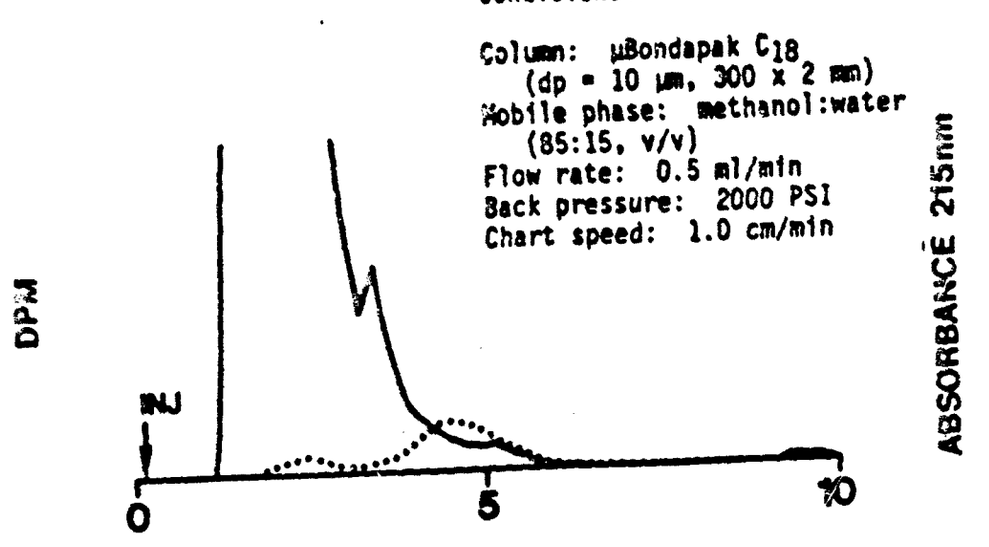

Figure 1A.

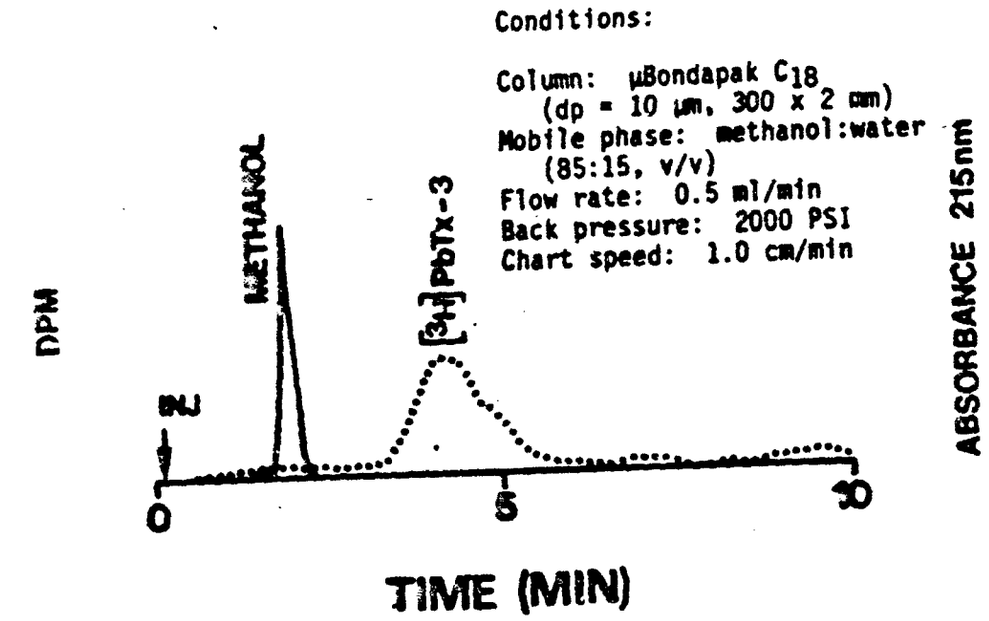

Figure 1. Chromatograms of: (A) receptor fluid which bathed human skin dosed with [^{3}H]PbTx-3 dissolved in DMSO and (B) methanol extract of skin 48 hr after it was topically dosed with [^{3}H]PbTx-3 dissolved in DMSO.

III. DISPOSITION OF [3H]PbTx-3 (A RED TIDE TOXIN) AFTER TOPICAL AND SUBCUTANEOUS APPLICATION IN WEANLING YORKSHIRE PIGS

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STATEMENT OF PROBLEM

In vitro methods are currently being used to determine the rate at which [3H]PbTx-3 penetrates through human skin. The correlation between *in vitro* and *in vivo* skin penetration values is reduced for more lipid soluble compounds (Hawkins and Reifenrath, 1984). Since PbTx-3 is a lipophilic compound (Baden, 1983), it is important to test the validity of using *in vitro* methods to measure the rate at which it penetrates skin. The purpose of this study was to compare the penetration of [3H]PbTx-3 through weanling Yorkshire pig skin using *in vitro* and *in vivo* methods. The specific objectives were to determine: (i)the amount of [3H]PbTx-3 which penetrates through excised pig skin, (ii)the amount of [3H]PbTx-3 which penetrates into the epidermis and dermis during the first 4 hours following application of [3H]PbTx-3 to the epidermal surface of excised pig skin, (iii) the amount of [3H]PbTx-3 excreted following topical and

subcutaneous application to pigs, and (iv) the distribution of [3H]PbTx-3 into muscle, liver, kidney and spleen following subcutaneous application to pigs.

BACKGROUND

PbTx-3 is one of a group of brevetoxins produced by the algae Ptychodiscus brevis. Blooms of this algae are referred to as red tide. People swimming during 'red tides' sometimes experience local dermal toxic effects which include skin irritation and itching (Ellis, 1985). These observations indicate that brevetoxins can at least penetrate through the layers of the stratum corneum, into the viable epidermis to cause toxic effects. It is not known if brevetoxins can penetrate skin and cause systemic effects in man.

RATIONALE USED IN CURRENT STUDY

In this study, weanling pig skin was used as a model for human skin because it has permeability properties similar to those of human skin (Reifenrath, Chellquist, Shipwash, Jederberg, and Krueger, 1984). In vitro measurement of PbTx-3 penetration through pig skin was first done with glass flow-through diffusion cells. In vivo determination of skin absorption was determined by mathematical comparison of the excretion of radioactivity following topical versus subcutaneous administration of [3H]PbTx-3. The parenteral administration is assumed to be 100% systemically absorbed and it

is distributed throughout the body and excreted (Feldmann and Maibach, 1970). In vivo skin absorption was also determined by the 'analysis of difference' method. This method involved excising the skin at the site of application and measuring the amount of radioactivity remaining in this tissue and in the barrier at the end of the exposure period.

It is widely accepted that the rate of penetration of compounds through skin is characterized by an initial lag phase(Guy and Hadgraft, 1985). This is the period of time required to reach steady state. Several techniques have recently been developed which enable the measurement of appearance of penetrant in dermal vasculature. The isolated perfused porcine skin flap (IPPSF) is an in vitro technique in which appearance of penetrant/metabolites in dermal vasculature is monitored by sampling media which perfuses the flap (Riviere, Bowman, Monteiro-Riviere, Dix, and Carver, 1986). The rat-human skin flap system (RHSFS) is an in vivo method in which appearance of penetrant in the dermal vasculature is measured by sampling the femoral vein draining the flap (Wojciechowski, Pershing, Huether, Leonard, Burton, Higuchi, Krueger, 1986). One advantage of these techniques is that the penetration of compounds can be directly assessed. In vivo skin absorption methods which calculate absorption by comparing excretion following topical versus parenteral administration are not able to determine the time course of penetration during the initial exposure period. Results from skin penetration studies using the RHSFS indicate that the lag phase is

shorter for some compounds than originally observed from previous studies (Reifenrath personal communication). One of the disadvantages of the RHSFS and IPPSF methods is that they are labor intensives and expensive to perform (Pershing and Krueger, 1987). A technique has been developed in which the penetration of compounds into the layers of the skin during the initial period of exposure can be easily determined. This method involves applying the compound to the epidermal surface of skin (in vivo or in vitro), using a dermatome to section the skin and then measuring the compound in each layer of the skin. This method was used to determine the early time course of [3H]PbTx-3 penetration into excised pig skin.

EXPERIMENTAL METHODS

Chemicals: (42-3H)PbTx-3 and unlabeled PbTx-3 were obtained from Dan Baden, University of Miami, Miami, FL.The radiolabeled PbTx-3 had a specific activity of 14.4 Ci/mmol. The radiochemical purity was determined by high performance liquid chromatography (HPLC). The HPLC methods involved using a uBondapak C18 column (Waters, Milford, MA), mobile phase of methanol:water (85:15,v/v),flow rate of 0.5 ml/min, u.v. detection was monitored at 215 nm and radioactivity was monitored with a FLO-ONE, Packard, Tampa, FL. The radiochemical purity was determined to be greater than 98%.

In vitro skin penetration study: Split thickness (1 mm) skin was excised from the upper back of a female weanling Yorkshire pig and mounted on glass flow-through diffusion cells. Skin samples were obtained from pigs sacrificed as part of another currently approved protocol in the Division of Cutaneous Hazards. The skin surfaces were each dosed with 0.3 ug/cm2 [3H]PbTx-3 (5 uCi) dissolved in 6 ul dimethylsulfoxide (DMSO), using a Hamilton microliter syringe with a blunt tip. An occlusive glass dome was placed over the epidermal surface during the experiment. Accumulated radioactivity (PbTx-3 and metabolites) in the receptor fluid was measured by collecting receptor fluid at hourly intervals and analyzing radioactivity in each sample with standard liquid scintillation (LSC) techniques. At the end of the experiment (24 hr after dosing epidermal surface) the epidermal surfaces were washed with soap and water (using Q-tips) to determine the amount of toxin remaining on the skin surface. The glass domes which covered the epidermal surfaces during the experiment were rinsed with methanol and radioactivity analyzed.The area of the skin dosed with toxin was then cut away from the perimeter portion of the skin. These two portions of each skin disk were extracted separately in order to determine if part of the applied dose had diffused to the perimeter of the skin disk and was unavailable for diffusion through the skin, into the receptor fluid. Each skin disc was then extracted twice with 10 ml of methanol. The radioactivity in receptor fluid, skin wash, skin extracts, and occlusive covering was then summed to determine total recovery of the applied dose.

In vitro distribution of toxin into layers of skin: Full-thickness skin was obtained from 2 weanling Yorkshire pigs. The skin was cut into segments approximately 6 cm x 12 cm and placed dermal side down on stainless steel trays which were lined with gauze pads soaked with RPMI media (GIBCO).The areas to which the toxin was to be applied was outlined with an alcohol marker by using a template measuring 2 cm x 4 cm. The toxin (3.1 ug [3H]PbTx-3 dissolved in 24 ul DMSO) was applied to each epidermal surface. The dosed skin segments were divided into 4 groups which were incubated at room temperature (22 degrees C) for different periods of time (15 min, 1 hr, 2 hr and 4 hr). The distribution of radiolabeled toxin into layers of the skin was determined by first swabbing the surface of the skin with soap, water and cotton Q-tips to remove compound which remained on the skin surface. The skin was then layered into epidermis, dermis, and subcutaneous sections by the use of an electric dermatome (Brown Electrodermatome Model 901, Zimmer-USA, Warsaw, Ind.) with a blade setting of 7. As each successive layer was removed, the skin layer was placed in a scintillation vial which contained 10 ml of methanol. The dermatome head cutting surface was swiped with methanol moistened cotton to remove any residual compound. The cotton swab was then extracted with methanol and the amount of compound measured was added to the proper level of skin from which the swipe was taken. Sectional layers of skin and dermatome swipes were taken until the dermis had been removed and the subcutaneous connective tissue exposed. The remaining skin bulk was referred to simply as the subcutaneous layer. The skin was left in methanol for approximately 24 hr after which time the

skin was removed and placed into an empty vial. An aliquot of the methanol extract of each skin section was then mixed with counting solution (Optifluor, Packard Instrument Co., Downers Grove, IL) for LSC. The remaining portion of the methanol extracts were individually evaporated to dryness under a steady stream of nitrogen and immediately dissolved in 100 ul methanol. The radiochemical composition of these samples was determined with HPLC (see Chemicals section for method).

A control group was run in order to determine if radioactivity on the dermatome cutting head was contaminating subsequent skin sections and thus confounding the results. This was done by placing skin segments on a tray dermal side down (as described above). The epidermal surfaces of skin in the treated group were dosed with [3H]PbTx-3 dissolved in DMSO and the skin segments in the other group were dosed only with the vehicle (DMSO). All of the skin segments were incubated for 45 min and then the skin surfaces were decontaminated as described above. The dermatome was then used to: (i)remove the epidermis of the skin segments which were dosed with DMSO only and (ii)remove the epidermis of the skin segment which had been dosed with [3H]PbTx-3. The dermatome head was then decontaminated with methanol soaked cotton swabs and was then used to remove a section of the dermis from the skin segment which had been dosed with DMSO only. This procedure was used to determine the amount of toxin which was carried over from slicing the epidermal layer (which would have the highest level of radioactivity) to the dermal layer.

In vivo disposition study: The in vivo skin absorption study was divided into two phases. The first phase was to determine the disposition (appearance of radiolabel in urine, feces, liver, kidney, spleen and muscle) following parenteral administration of [3H]PbTx-3 to 3 pigs. The second phase was to determine the clearance of the radioactivity following topical administration of [3H]PbTx-3 to 3 additional pigs. The pigs used for this portion of the study were female weanling Yorkshire pigs weighing between 9 and 17 Kg and were obtained Boswell Laboratory Animals, Corcoran, CA.

The parenteral study involved dosing the pigs subcutaneously with 18 uCi radiolabeled PbTx-3 (1.1 ug) dissolved in 0.5 ml propylene glycol. The pigs were then housed in stainless steel metabolism cages for 3.25 days. During this time urine and feces were collected separately twice a day. The pigs were then killed by intramuscular injection of a mixture of xylazine and ketamine, followed by an intravenous injection of T-61 after the pigs were sedated. The liver, kidney, spleen and piece of muscle (pectoralis) were removed from each pig. Radioactivity in urine samples was determined by mixing a aliquot (1 ml) of each urine sample with Optifluor for LSC counting. The feces were freeze-dried and samples placed in zip-lock polyethylene bags and ground to a fine powder with a teflon rolling pin. A 0.2 gm aliquot was taken from each sample, and mixed with 0.2 gm cellulose powder and oxidized in a Packard Model 306 Sample Oxidizer (Packard Instruments, Des

Plains, IL). Radioactivity in these vials was then determined by LSC. The liver, kidney, spleen and piece of muscle were separately lyophilized and converted to powder. Approximately 0.2 gm of sample and 0.2 gm of cellulose powder were burned on the Packard Oxidizer.

The topical studies were done by applying 20 uCi radiolabeled Pbtx-3 (0.4 ug/cm2) to the upper back of pigs. The application site had a surface area of 25 cm2. A non-occlusive protective patch was placed over the application site. The patch was constructed from a foam pad (Reston Foam Pad, 3M Company, Minneapolis,MN). Rectangles were cut out of two pieces of foam pad, each rectangle was larger than the perimeter of the application site. A nylon screen was then sandwiched between the two foam pads and held together with glue. The patch was positioned on the pig so that the screen was over the application site. The patch was then held in place by wrapping surgical tape around the perimeter of the pad and around the torso of the pig. This patch kept the application site from coming in contact with the metabolism cage and prevented radioactive exfoliated skin from falling into the collection device for urine and feces. The pigs were then housed in metabolism cages for 4.25 days and urine and feces collected as described above. On day 2 the protective patches were removed and replaced with new patches. On day 3 the patches were removed and the site of application decontaminated with cotton moistened with soap and water. The radioactivity in the patches and cotton swabs was determined by extracting them separately with methanol and analyzing radioactivity in each extract. The pigs were then killed

with an intramuscular injection of acepromazine followed by an intravenous injection of T-61 after the pigs were sedated. The skin from the application site was then removed and an electric dermatome used to section the skin into epidermis, dermis, and subcutaneous fat (as described in section: In vitro distribution of toxin into layers of skin).

RESULTS AND DISCUSSION

In vitro skin penetration study: The rate of penetration of [3H]PbTx-3 through excised pig skin is shown in Figure 1. The rate is expressed as the nanograms of PbTx-3 equivalents which accumulated in the receptor fluid per hour. These calculations were based on the amount of radioactivity in the receptor fluid and do not take into account that some of the radioactivity may have been associated with metabolites of PbTx-3. In vitro studies on the penetration of [3H]PbTx-3 through human, guinea pig and monkey skin indicate that PbTx-3 is not metabolized by excised skin (Kemppainen, Mehta, and Clark, 1988). The recovery of the applied dose at the end of the experiment (24 hr after applying dose) is shown in Figure 2. Only 0.3% of the total dose was recovered in the receptor fluid. However, 37% of the dose had penetrated into the skin. Part of the dose within the skin is in the stratum corneum and would probably be exfoliated if it was a in vivo situation. However, part of the dose in the skin is probably in the dermis and would be available for absorption into systemic circulation. In future studies we plan to use a electric dermatome

to separate the epidermis and dermis at several time periods in order to determine the rate at which toxin is penetrating into the layers of the skin.

In vitro distribution of toxin into layers of skin: The penetration of [3H]PbTx-3 into layers of excised pig skin during the initial exposure period (15 min to 4 hr) is shown in Figure 3. The amount of toxin is greatest in the epidermis, followed by the amount in the dermis, and lowest in the subcutaneous fat. The pattern of toxin accumulation in each layer is similar: initial concentration (15 min) decreases by 1 hr, increases by 2 hr, and then decreases again by 4 hr. The peak concentration at 2 hr may be due to the vehicle (DMSO) temporarily enhancing penetration. DMSO is a hydroscopic compound which may act to draw water out of the skin, thus diluting the DMSO which remains on the skin surface and diminishing its penetration enhancing effects.

Chromatographic analysis of the methanol extracts of the skin layers indicated that [3H]PbTx-3 was not metabolized by the excised pig skin.

Results from the control study indicated that radioactivity from the dermatome head was not contaminating subsequent skin sections and not confounding the results. The results indicated that less than 0.16% of the radioactivity in the treated skin layers was due to contamination from the dermatome head.

In vivo disposition studies: The cumulative excretion of radioactivity following subcutaneous application of [3H]PbTx-3 to pigs is shown in Figure 4. The total excretion of radioactivity in the urine and feces (expressed as percent of dose) was 10.7 and 8.9, respectively. The rate of appearance of radioactivity in the feces and urine had not begun to decrease by the end of the collection period (3.2 days). This is in contrast to the kinetics of excretion of radioactivity in rats following intravenous administration of [3H]PbTx-3 (Poli and Pace, personnel communication). In the rat the rate of excretion had begun to decrease by approximately day 2, at which point approximately 60% of the dose had been excreted.

The disposition of radioactivity into the liver, kidney, spleen, and muscle is shown in Figure 5. The largest portion of the recovered dose was in the muscle.

The cumulative excretion of radioactivity following topical application of [3H]PbTx-3 in pigs is shown in Figure 6. The total excretion of radioactivity in the urine and feces (expressed as percent of the dose) was 0.47 and 1.2, respectively. Mathematical comparison of the excretion of radioactivity following parenteral and topical administration indicates that 8.5% of the dose was absorbed. However, the accuracy of these calculations is reduced because the rate of excretion of radioactivity in the pigs had not begun to decrease when the experiment was terminated. The amount of toxin which was percutaneously absorbed was also calculated by

the 'analysis of difference' method. This was done by summing the amount of radioactivity (expressed as % of dose) extracted from:the patches (39.4%), the cotton used to decontaminate the site of application (34%), and the skin at the site of application (3.4%). The total amount of radioactivity remaining at the site of application or in the patch was 77%. Thus the analysis of difference method indicates that 23% of the dose was absorbed percutaneously. It is difficult to compare the amount of dose which penetrated the pig skin *in vitro* (0.3%) with the dose which penetrated the skin *in vivo* (8.5% or 23%, based on excretion method or analysis of difference method). This is because the exposure period in the *in vitro* study was for 24 hours and the exposure period in the *in vivo* study was for 48 hr. In order to make this comparison it is necessary to repeat the *in vitro* study and increase the exposure period to 48 hr. In order to accurately determine *in vivo* skin absorption of [3H]PbTx-3 it is necessary to repeat the *in vivo* study and monitor excretion of radioactivity for a longer period (until excretion of radioactivity reaches background levels).

CONCLUSION

[3H]PbTx-3 does penetrate into and through pig skin. *In vitro* findings indicate that as little as 0.3% of the dose penetrated into the receptor fluid but 37% of the dose penetrated into layers of the skin 48 hr after topical application. Analysis of the skin layers indicated that 7.2%, 2.8%, and 0.5% of the dose had

penetrated into the epidermis, dermis, and subcutaneous fat (respectively) by 2 hours after topical application. Preliminary in vivo findings indicate that between 8% and 23% of the dose was percutaneously absorbed. It is likely that these in vivo findings over estimate percutaneous absorption since topical application of a dose of brevetoxin (which was 5 - 10 times greater than LD50 by parenteral route) did not cause lethality in guinea pigs(Wannemacher, Bunner, and Dinterman, 1988).

These results suggest that PbTx-3 readily penetrates into skin but possibly because its lipophilicity it does not readily penetrate into receptor fluid. These findings indicate that in future studies on skin penetration of low molecular weight compounds it is important to determine the amount of toxin which penetrates into the layers of skin and would be available for systemic circulation.

Footnote

In the above discussion several experiments are discussed which would clarify the results. We do not plan to do those additional experiments with [3H]PbTx-3 and pig skin. Instead, we plan to use these experimental techniques to determine the in vitro and in vivo skin penetration of other low molecular weight toxins (saxitoxin and lyngbyatoxin A) by using excised human skin and intact guinea pigs or hairless mice.

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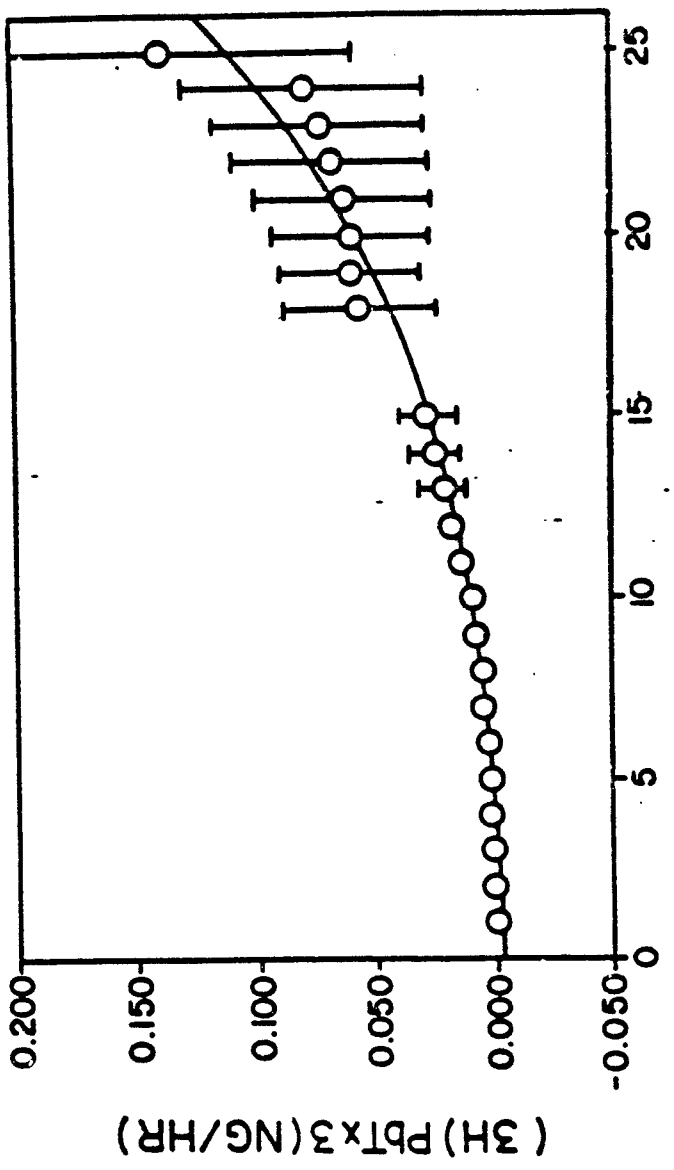

Figure 1. Penetration of [^{3}H]PbTx3 through excised pig skin (vehicle: DMSO)

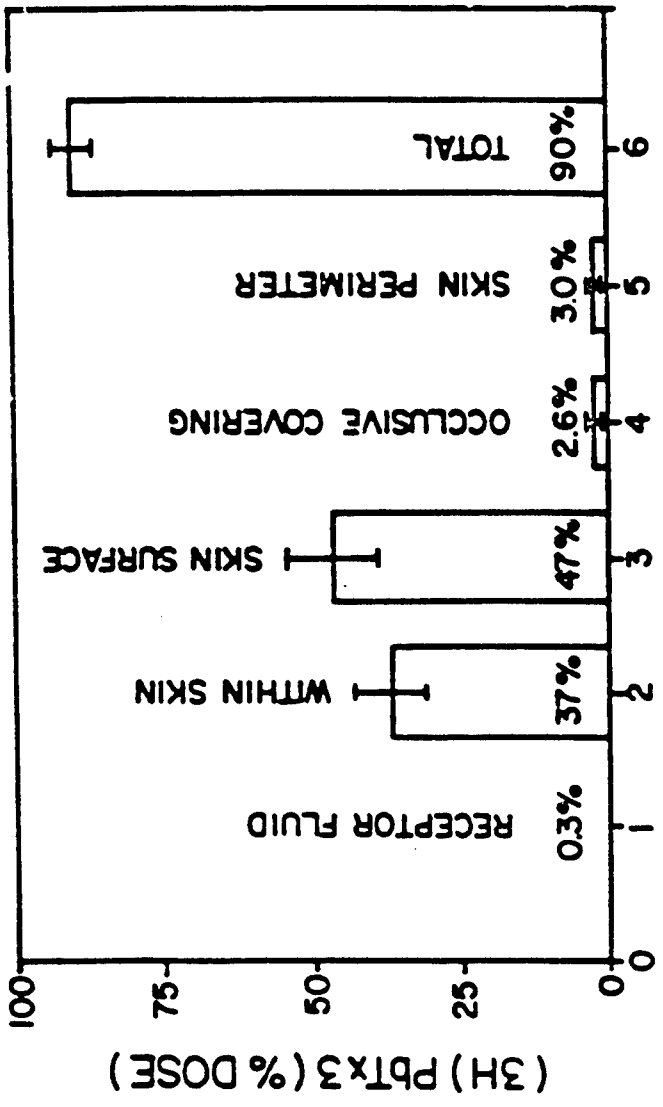

Figure 2. Recovery of [^{3}H]PbTx3 from diffusion cells.

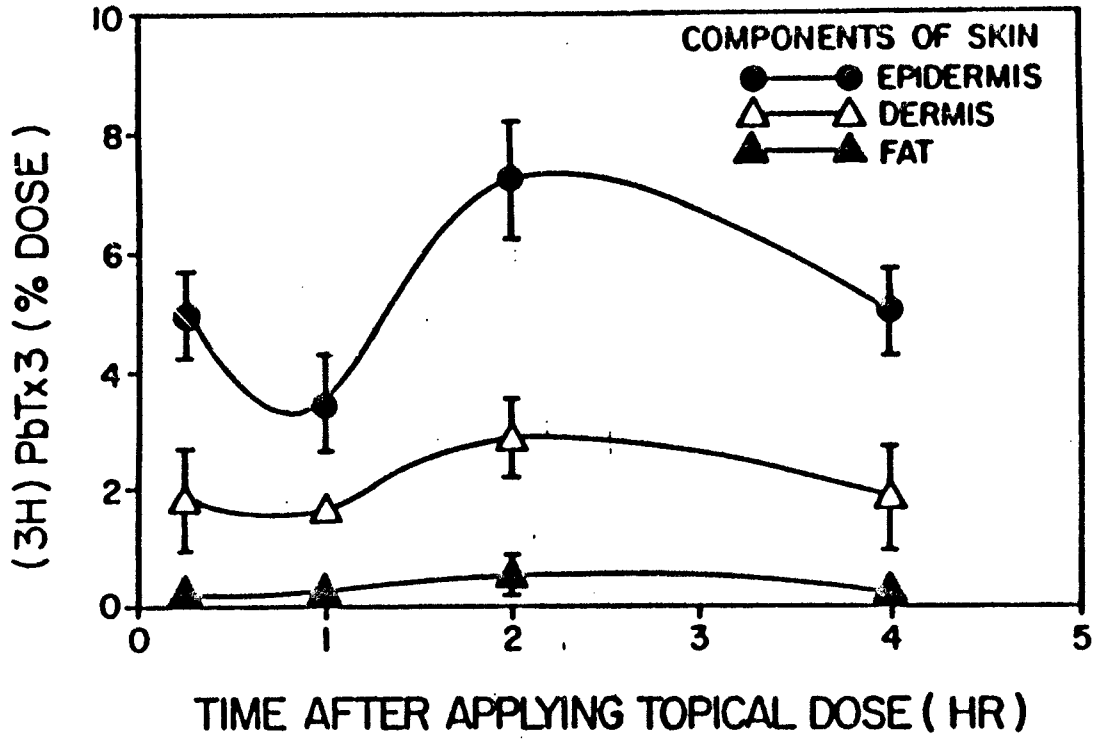

Figure 3. Penetration of [^{3}H]PbTx3 into layers of excised pig skin.

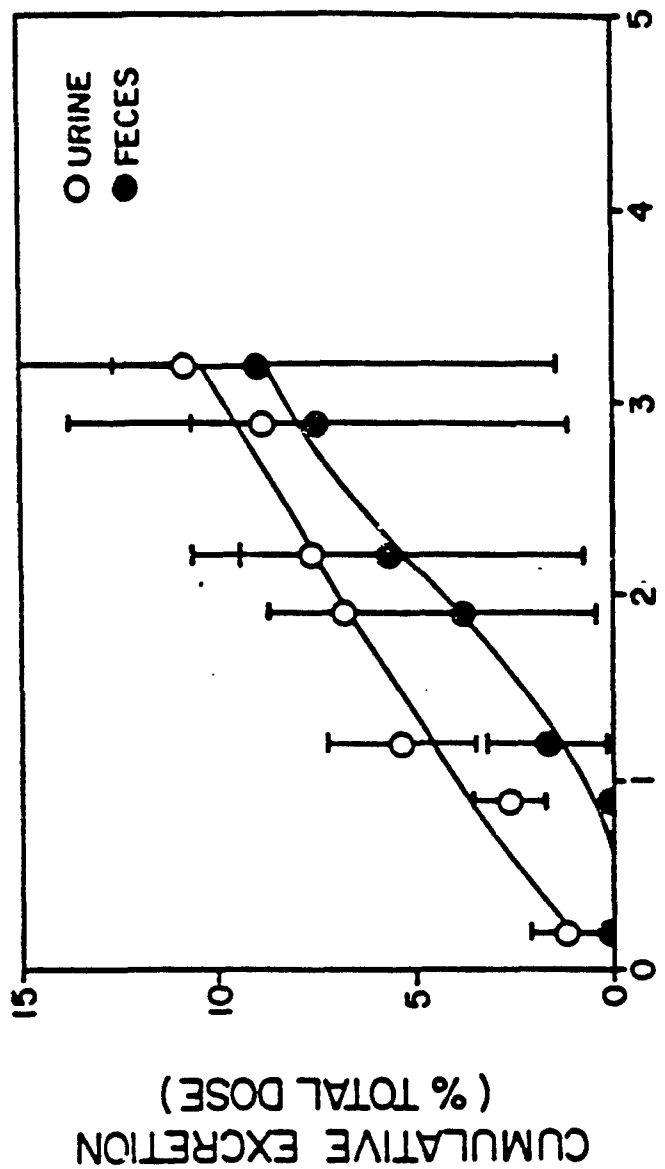

Figure 4. Excretion of [^{3}H]PbTx3 following subcutaneous application to weanling pigs.

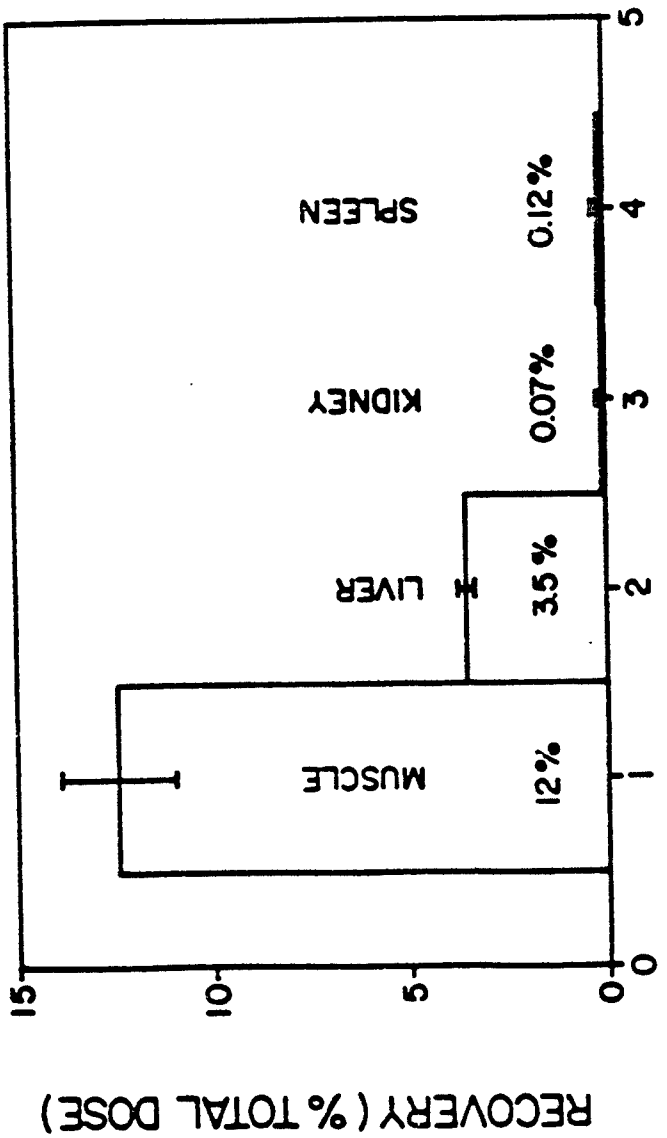

Figure 5. Recovery [^{3}H]PbTx3 in organs following subcutaneous application to weanling pigs.

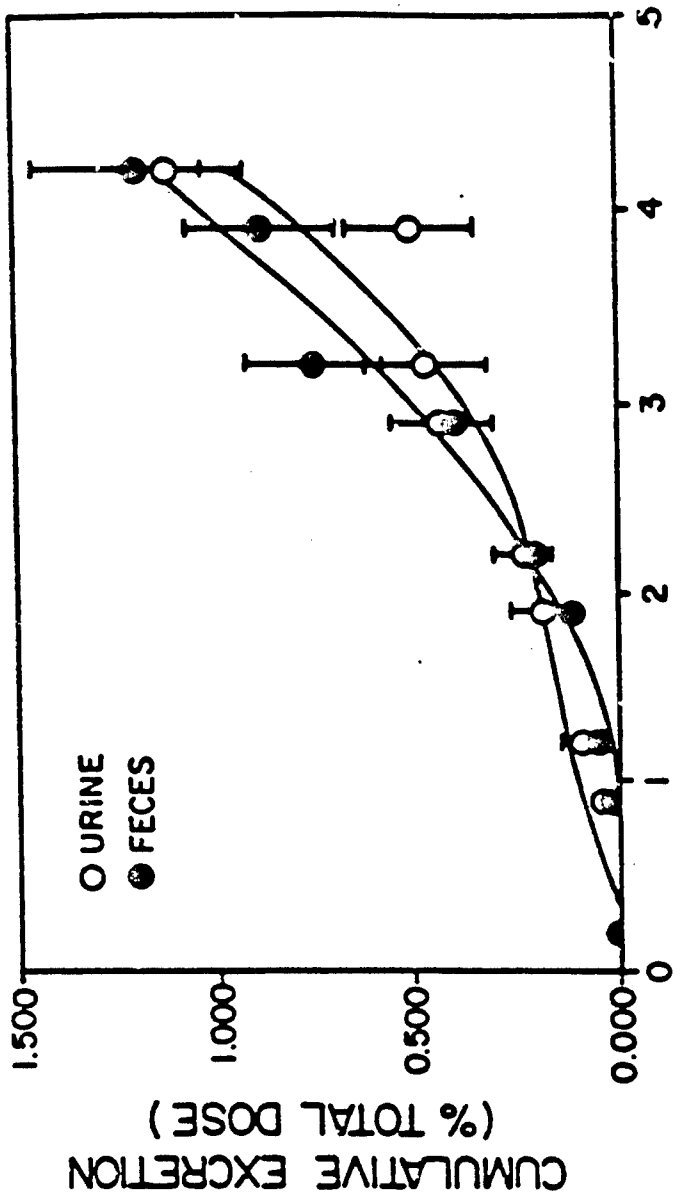

Figure 6. Excretion of [^{3}H]PbTx3 following topical application to weanling pigs.

IV. IN VITRO PERMEABILITY OF MONKEY BUCCAL MUCOSA AND SKIN TO TRITIATED WATER (THO) AND [3H]PbTx-3 (RED TIDE TOXIN).

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STATEMENT OF PROBLEM

The primary purpose of this study was to determine the penetration of THO and [^{3}H]PbTx-3 through excised monkey buccal mucosa. According to the contract, these studies were planned to be conducted in excised human buccal mucosa, but due to difficulty in obtaining excised human buccal mucosa, it was decided to conduct the above study in excised monkey tissue. Monkey tissue was used because the animal models most predictive of percutaneous absorption in man are pig and monkey (Ronald C. Wester and Patric K. Noonan,1980). Penetration of THO through monkey buccal mucosa and skin was studied so that the permeability of monkey buccal mucosa could be compared to permeability of dog and pig buccal mucosa (William R. Galey et al. J Invest Dermatol 67:713-717, 1976).

BACKGROUND

Brevetoxin (PbTx-3) is one of a group of toxins produced by a marine dinoflagellate Ptychodiscus brevis, fig 1. Blooms of this

dinoflagellate are referred to as red tide. Blooms of P.brevis (formerly known as Gymnodinium breve) are responsible for massive fish kills in the Gulf of Mexico and along Florida coast (Mende and Baden,1978). The red tides are also implicated in human intoxication resulting from ingestion of contaminated shellfish or inhalation of seaspray aerosols. One of the earliest records of human respiratory irritation associated with red tide was provided by Taylor (1917) during his observations of a red tide outbreak along the Florida's west coast. Similar observations were later noted along Florida's west coast by Lund. The relationship between respiratory irritation and aerosolized dinoflagellate products was observed by Woodcock (1948) during a severe red tide episode at Venice, Florida, in 1947. When seawater containing the red tide organisms was sprayed as an aerosol into the nose and throat of volunteers, coughing and a burning sensation resulted, similar to that experienced along local beaches. The organism was identified as G. breve by Davis (1948). Through this and several other experiments, Woodcock (1948) concluded that "human respiratory irritation is associated with the presence in the inhaled air of dinoflagellates".

The lipid soluble toxins produced by P.brevis are of two types, one of which is hemolytic and the other neurotoxic (Padilla et al., 1979). The neurotoxin has a unique structure of a cyclic polyether (Fig. 2) with a molecular weight of 898 (Lin et al., 1981; Chou and Shimizu, 1982). Most of the in vivo studies undertaken utilizing bioactive compounds from P.brevis have dealt with the effects of neurotoxin administration (Baden et al.,

1979,1981). The *in vivo* physiological effects of *P.brevis* are both gastrointestinal and neurological in nature (D.G. Baden,1983). Significant *in vitro* investigations have been done to determine the mechanism of action (Sanser et al, 1972; Abbott et al,1975; Kim et al,1978).

EXPERIMENTAL METHODS

MATERIALS

THO was generously provided by Mr. John King (Department of Safety and Nuclear Medicine, Auburn University, AL). [^{3}H]PbTx-3 was purchased from Dr. D.G. Baden (University of Miami, Fl). [^{3}H]PbTx-3 supplied was more than 99% pure (fig.3), specific activity was 12.3 to 14.3 Ci/mmol. High pressure liquid chromatography (HPLC) was performed with a unit from Waters Associates, Milford, MA, which consisted of a U6K Injector, Model 740 Data Module and Model 712 Waters Intelligent Sample Processor (WISP). Reverse phase chromatography was used. Either Waters uBondapack C18 steel column (10um, 3.9 mm x 36cm) or Hypersil ODS column (5 um, 50 x 4.6 mm, Keystone Scientific, Inc.,State College, PA) was used. Eluant was methanol :water (85:15). Radioactive flow detector (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL) was attached to Waters HPLC system to analyze the radiolabelled brevetoxin, Flo- Scint (Radiomatic instruments and Chemical Co. Inc.) was the scintillation fluid used for radiochromatographic analysis.

To verify the identity of brevetoxin peak: Eluant was

collected from 4 to 6 min. (retention time of brevetoxin) with a fraction collector (ISCO Inc.,Lincolon, Nebraska). Collected eluant was dried down under nitrogen stream and residue was dissolved in normal saline. Female ICR mice weighing 22.75 ± 2.5 gm (mean ± SD), were dosed i.p. The dose (4 ug/20 gm) was dissloved in normal saline and the volume was 60 to 74 ul. For the control group , 10 ul of methanol was injected on HPLC , eluant was collected at the same retention time as that of [^{3}H]PbTx-3 (4-6 min.), evaporated to dryness and was dissolved in normal saline. Number of mice in each group was 2. Food and water were provided to both the treated and control group ad libitum. No effect was observed in control group. After 2 to 4 hr of dosing, treated group mice were depressed, did not approach food and water, urination was increased. After 24 hr one treated mouse died, surviving mouse was fully recovered. LD_{50} was consistent with reported toxicity (Baden et al.,1981).

Stability of [3H]PbTx-3 in the receptor fluid was evaluated by mounting Teflon discs, instead of tissue discs on the diffusion cells. The receptor fluid was dosed with 120 -160 ng PbTx-3/100ul of water. The cells with Teflon discs were incubated along with cells with tissue discs, in the environmental chamber (36°C). Samples from receptor fluid were analyzed as described below.

Monkey buccal mucosa and skin were obtained from primate centers in Wisconsin (Wisconsin Regional Primate Research Center, Madison, Wisconsin), Louisiana (Delta Primate Center, Covington, Louisiana), California (California Regional Primate Research Center. California), Washington (Washington Regional Primate Research Center, Seattle, Washington). The monkeys were sacrificed

as part of other ongoing studies. Collected tissues were packaged with wet ice and shipped by overnight delivery.

METHODS

Loose fat and connective tissue were removed from the inner surface (dermal side) of monkey buccal mucosa and skin. After measuring the thickness, discs of excised tissue, each with a surface area of 2.8 cm^2, were mounted on static teflon diffusion cells (fig.4). To support the delicate buccal mucosa, some of the buccal mucosal discs were placed in between two layers of large pore nylon or teflon filter membranes (Cole-Parmer, Chicago, IL). Some of the skin discs were also placed in between these membranes in order to determine if the nylon or teflon membranes interfere with penetration. Dermal (inner) and epidermal (outer) surfaces were bathed with Hanks Balanced Salt Solution with HEPES buffer and gentamycin (HBSS) and cells were incubated in refrigerator (4°C) overnight to fully hydrate the tissue. HBSS was used as receptor fluid and was bubbled with O_2:CO_2 (95:5). The dissolved oxygen in the receptor fluid (for static type of diffusion cell) was increased to promote viability (Hawkins, personal communication). To accomplish this, the media was first degassed in a vacuum dessicator for approximately ten minutes using a vacuum pump. The media was then regassed by bubbling for approximately five minutes with O2:CO_2 (95:5) . At the time of dosing, HBSS on epidermal surface was replaced with dose (0.76 uCi of THO /2.8cm^2 or 371-412 ng [^{3}H]PbTx-3/2.8 cm^2, diluted in 2 ml of water). Epidermal surfaces were occluded and cells were incubated at 37°C for 24 hr. Sequential samples were drawn from dermal side (receptor fluid).

Receptor fluid samples from the cells dosed with THO, were drawn every 15 inutes for the first hr, every 30 minutes over next two and half hr, every one hr over next 5 hr, and then at the end of the experiment (24 hr). From the cells dosed with [3H]PbTx-3, receptor fluid samples were drawn every 30 minutes for the first 2 hr, every one hr over next 5 hr, every 2 hr over next 6 hr and then at the end of the experiment (24 hr). Receptor fluid sample size was 50 ul each. Samples from epidermal side were taken at 0 and 24 hr from the cells dosed with THO and at 0, 13 and 24 hr from the cells dosed with [^{3}H]PbTx-3. Temperature and relative humidity was recorded frequently during the entire length of experiment and was 36°C, 29±4% (mean ±SD), respectively.

At the end of the experiment, dose left on epidermal surface was collected and measured. Epidermal surfaces were washed with soap and water using Q-tips. To extract THO, Q-tips were extracted with 5 ml of water, tissue discs were extracted with 10 ml of water. To extract [^{3}H]PbTx-3, methanol was used instead of water. THO or [^{3}H]PbTx-3 was extracted by sonicating the vials containing Q-tips and tissue discs for 10 minutes. This was repeated once. Aliquots of receptor fluid, skin wash , skin extract and dose left on the epidermal surface were counted by Liquid Scintillation Analyzer, Packard Model 1500 Tri-Carb (Sterling, Virginia) to determine the recovery of dose applied. From the cells dosed with [^{3}H]PbTx-3, above mentioned aliquots were also injected on HPLC to determine the metabolism or breakdown of [^{3}H]PbTx-3.

RESULTS AND DISCUSSION

Percent of dose of THO which penetrated through tissue was plotted against time, refer to figures 5A and 5B. Christopher A. Squier et al (1985) calculated permeability coefficient (Kp) from values taken at 1 or 2 hr after reaching steady state. Steady state for THO penetration through buccal mucosa starts at 3.5 hr and continues through 12 hr and steady state of monkey skin starts at 7 hr and continues through 24 hr (fig 5A). Time before steady state represents the lag phase of penetration. Lag phase to penetration of THO was not observed through buccal mucosa with nylon or teflon membranes (fig 5 B). Figure 6A represents [^{3}H]PbTx-3 penetration through monkey buccal mucosa (DPM plotted against time) and skin. Figure 6B depicts the penetration of this compound through tissue discs placed in between the nylon membranes (expressed as percent of dose of [^{3}H]PbTx-3 penetrated against time). Steady state of buccal mucosa and skin to [3H]PbTx-3 penetration starts at 8 hr and 13 hr respectively, and continues through 24 hr (figure 6 A and B).

Formula used to calculate permeability coefficient (Kp) was

$$Kp = \frac{Q}{A.T.(Co-Cl)}$$

where Q is the net flux of compound, A is the area of the tissue exposed to compound, T is the time of exposure in sec, Co is concentration on epidermal side and Ci is the concentration on dermal side. Calculated Kp was normalized in terms of uniform thickness barrier of 750 um to facilitate comparison of permeability of tissues of different thickness. This was done by

multiplying the calculated value of Kp by thickness of tissue (permeability is inversely related to thickness) and arbitrarily dividing by 750. For example, a buccal mucosa of 1100 um thickness and Kp of 1295 x 10^{-7} cm/sec would be normalized by the following equation (1295 x 10^7) x (1100/750) = 1899 x 10^7 cm/sec.

Permeability of monkey buccal mucosa relative to skin was 15.6 and 8.8 times greater for THO and [3H]PbTx-3, respectively (see Table 1 and Table 2). These results were surprising because it was thought that since PbTx-3 is a lipophilic compound it would readily penetrate through buccal mucosa. The results of a report by Reifenrath et al (J. Pharm. Sci. 76:293,1987) indicate that during in vitro studies the dermis acts as an artificial reservoir for lipophilic compounds but not for hydrophilic compounds. In contrast, during in vivo studies the dermis does not act as a reservoir for either lipophilic or hydrophilic compounds. This is consistent with our finding that 24 hr after dosing the excised buccal mucosa and skin 46 and 24 % of [3H]PbTx-3 was within buccal mucosa and skin, respectively, but only 2 and 8 % of THO was residing in the buccal mucosa and skin, respectively (Table 3). Reifenrath (J. Pharm. Sci. 75:378- 381,1986) has found that there is a better correlation between the in vitro and in vivo skin penetration by lipophilic compounds when the in vitro skin penetration is expressed as the sum of the compound which has penetrated into the receptor fluid and which is within the dermis.

Permeability of monkey buccal mucosa to THO was 4 times greater than pig and 3.7 times greater than dog buccal mucosa.

Nylon membranes changed the penetration of THO and [^{3}H]PbTx-3

through monkey buccal mucosa by factors of 0.9 and 1.4 respectively. Permeability of monkey skin to THO was decreased by a factor of 1.1 by the nylon membranes, but was increased by a factor of 1.6 by nylon membranes (Table 1 and 2). We do not know why the presence of nylon membranes would change the penetration, but possibly some kind of reaction went on between the nylon membranes and tissues and toxins.

Metabolism: Analysis of the receptor fluid which had bathed buccal mucosa, indicated that 85 ±7% (mean ±sd) of activity was associated with [^{3}H]PbTx-3 and 15±5% (mean ±sd) of activity was associated with an additional peak (fig 7). Similar type of peaks were seen on chromatographic analysis of receptor fluid which had bathed the monkey skin dosed with [3H]PbTx-3 for 24 hr. This is probably due to slow breakdown of [^{3}H]PbTx-3 in aqueous receptor fluid, since [3H]PbTx-3 has been shown to break down in PBSA at the approximate rate of 7% per 24 hr. These results suggest [3H]PbTx-3 is not metabolised in excised monkey buccal mucosa and skin.

CONCLUSION

Pentration of THO and [^{3}H]PbTx-3 is faster through monkey buccal mucosa than through monkey skin, by factors of 15.6 and 8.8 respectively. In other words, there is greater difference to the penetration of hydrophilic compound (THO) than to the lipophilic compound ([^{3}H]PbTx-3), through monkey buccal mucosa and skin.

Nylon membranes had an effect on penetration of THO but negligible effect on penetration of [^{3}H]PbTx-3 through monkey

buccal mucosa and skin.

It is not necessary to use nylon membranes to support the delicate buccal mucosa, rather a little thicker layer of subcutaneous tissue acts as a better support.

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Table 1. Permeability of Monkey Buccal Mucosa and Skin to THO

Tissue (n)	Thickness (μm)	Kp (Mean ± SD x 10^{-7})	
		Calculated	Normalized[a]
Monkey Buccal Mucosa [B.M.] (3)	110 ± 43	1295 ± 84	1899 ± 123
B.M. with teflon[b] (1)	780 ± 41	2320 ± 500	2412 ± 519
B.M. with nylon[c] (2)	740 ± 41	2050 ± 762	2022 ± 751
Pig Buccal Mucosa	NP[f]	NP	464 ± 74[d]
Dog Buccal Mucosa	NP	NP	511 ± 61[e]
Monkey Skin (3)	783 ± 62	116 ± 10[b]	121 ± 8
Monkey Skin with nylon (4)	903 ± 62	84 ± 7	101 ± 8.4

[a]Normalized for thickness of 750 μm.

[b]Thickness of 2 layers of teflon membrane 250 μm.

[c]Thickness of 2 layers of nylon membrane 120 μm.

[d]Christopher A. Squier et al. J. Invest Dermatol. 84:176-179, 1985.

[e]Willium R. Galey et al. J. Invest Dermatol. 67:713-717, 1976.

[f]NP = not published.

Table 2. Permeability of Monkey Buccal Mucosa and Skin to [^{3}H]PbTx-3

Tissue (n)	Thickness (μm)	Kp (Mean ± SD x 10^{-7})	
		Calculated	Normalized[a]
Monkey Buccal Mucosa (8)	750 ± 41	417 ± 200	417 ± 200
Buccal Mucsa with nylon (2)	740 ± 41	577 ± 168	569 ± 165
Moneky Skin (2)	700 ± 18	50 ± 24	47 ± 22
Monkey Skin with nylon	903 ± 62	62 ± 11.0	75 ± 13

[a]Normalized for thickness of 750 μm.

Table 3. Distribution of the Compound 24 hr after applying the dose.

Compound	Tissue (n)	Recovery of applied dose (expressed as % dose)			
		Receptor Fluid	Within Tissue	Epidermal Surface	Total Recovered
THO	Buccal Mucosa (3)	44.3 ± 2.8[a]	1.8 ± 0.2	30.4 ± 1.7	77.6 ± 4.5
	Monkey Skin (5)	8.3 ± 3.9	7.7 ± 4.3	60 ± 16.8	71.1 ± 16
PbTX-3	Buccal Mucosa (4)	4.2 ± 2.1	45.7 ± 18.0	41.9 ± 13.6	89.6 ± 14.6
	Monkey Skin (3)	0.7 ± 0.4	23.6 ± 8.6	68.7 ± 4.5	92.6 ± 6.2

[a]Mean ± SD

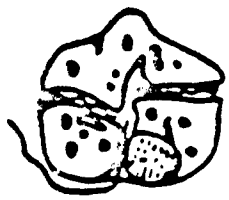
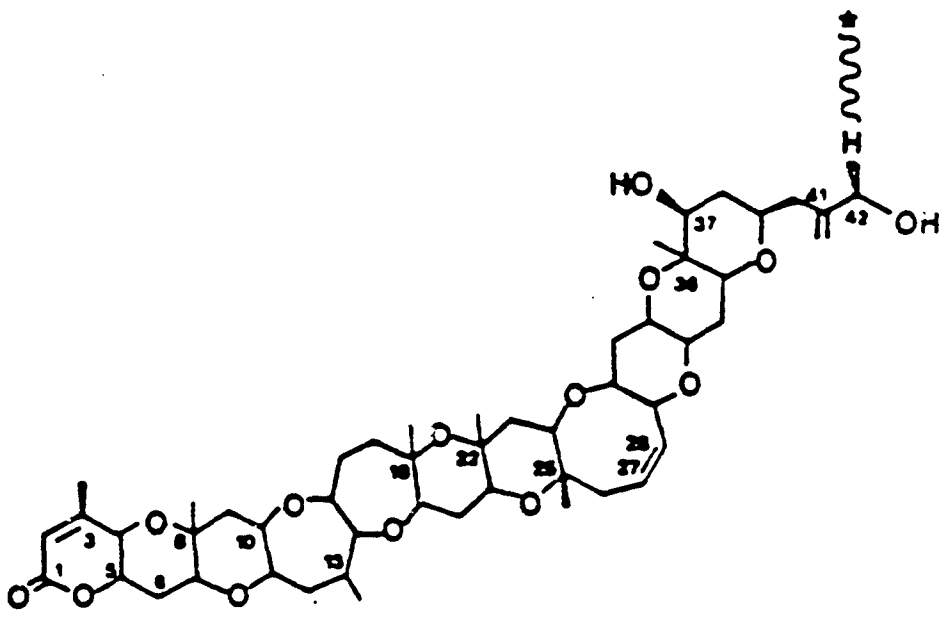


Figure 1. Dinoflagellate P.brevis.



Brevetoxin

[42-^{3}H]PbTx-3

Figure 2. Structure of [^{3}H]PbTx-3.

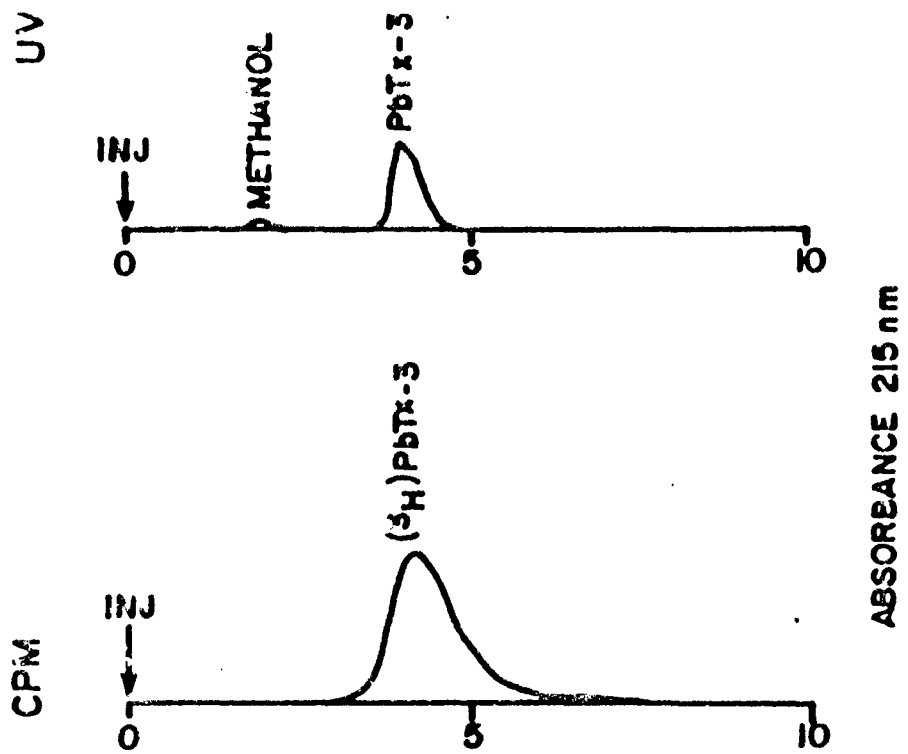


Figure 3. [^{3}H]PbTx-3 Chromatogram. Chromatographic conditions: Column - uBondapak C18 (10 um, 300 x 2 mm); mobile phase - methanol : water (85:15, v/v); flow rate - 0.5ml/min.; back pressure - 2000 PSI; chart speed - 1.0 cm/min.

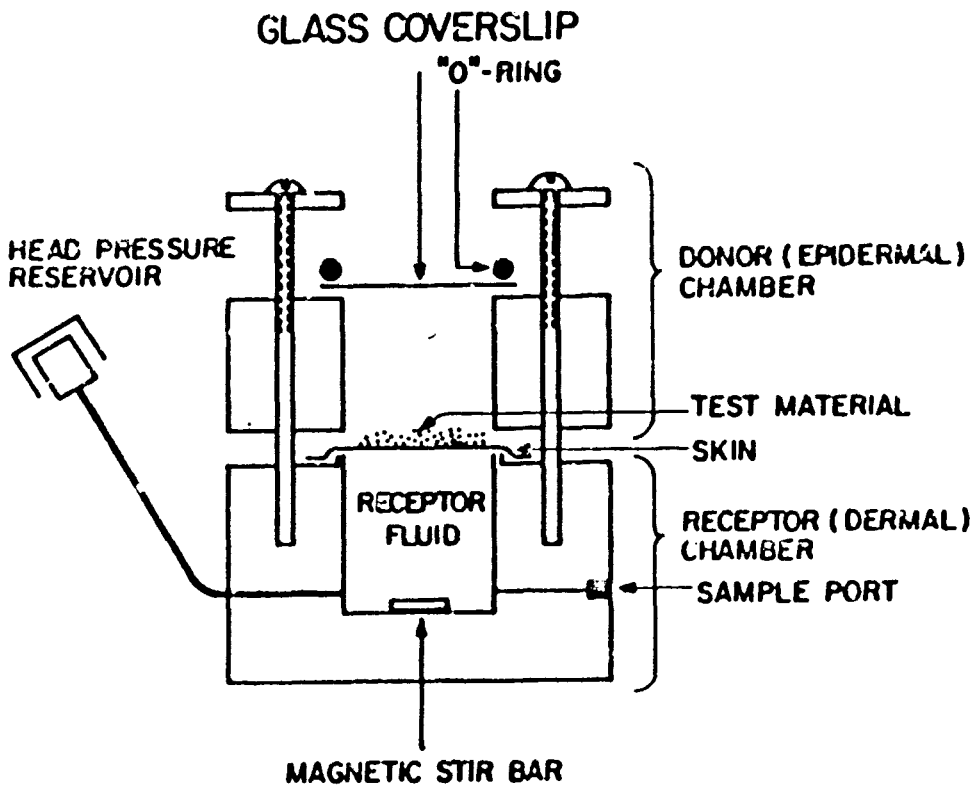


Figure 4. Static diffusion cell used for skin penetration studies.

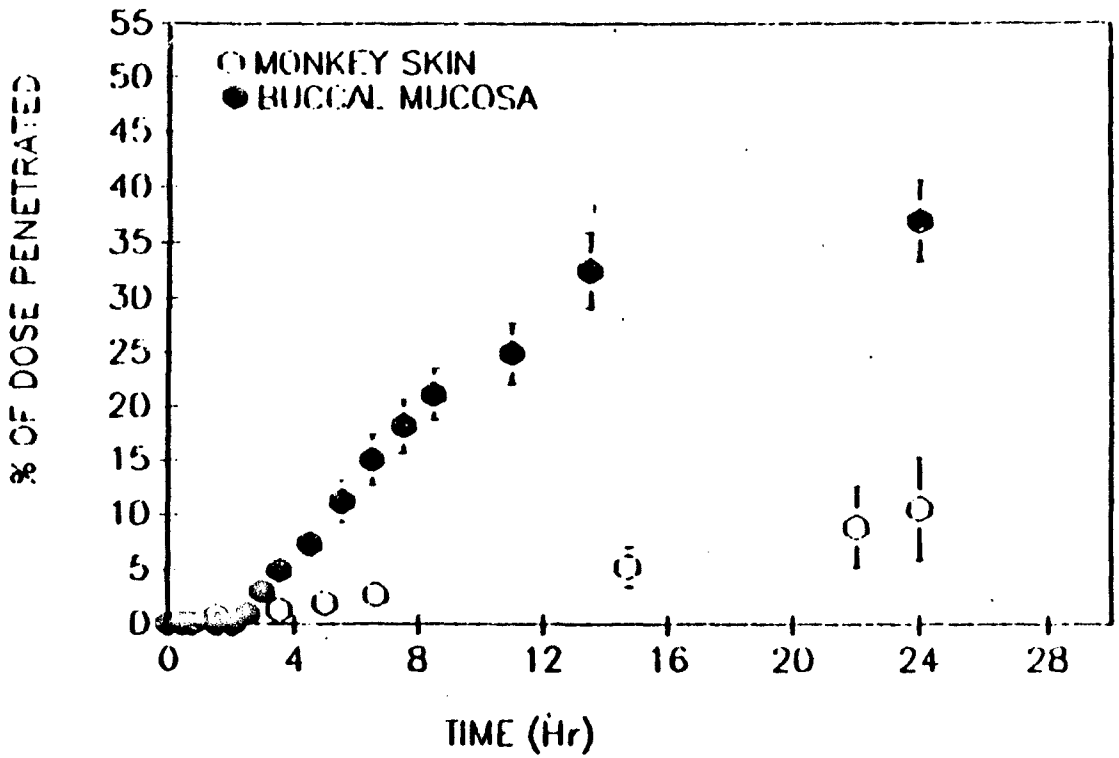


Figure 5A. Penetration of tritiated water (THO) through excised monkey buccal mucosa and skin (expressed as cumulative percent dose).

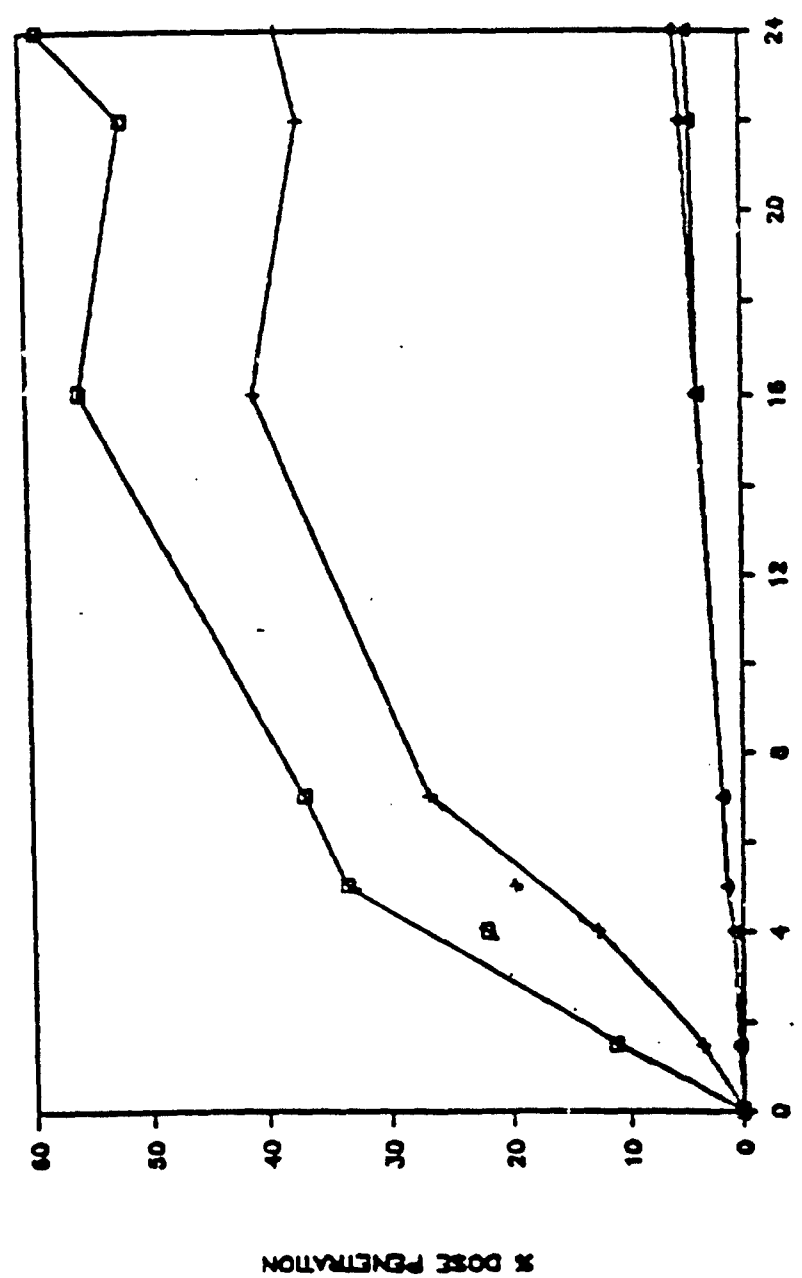


Figure 56. Penetration of tritiated water (THO) through discs of excised monkey buccal mucosa and skin, some of which had been sandsiched between large pore nylon and teflon membranes for support. BMT = buccal mucosa between teflon membranes, BMN = buccal mucosa between nylon membranes, MS = monkey skin and MSN = monkey skin between nylon membranes.

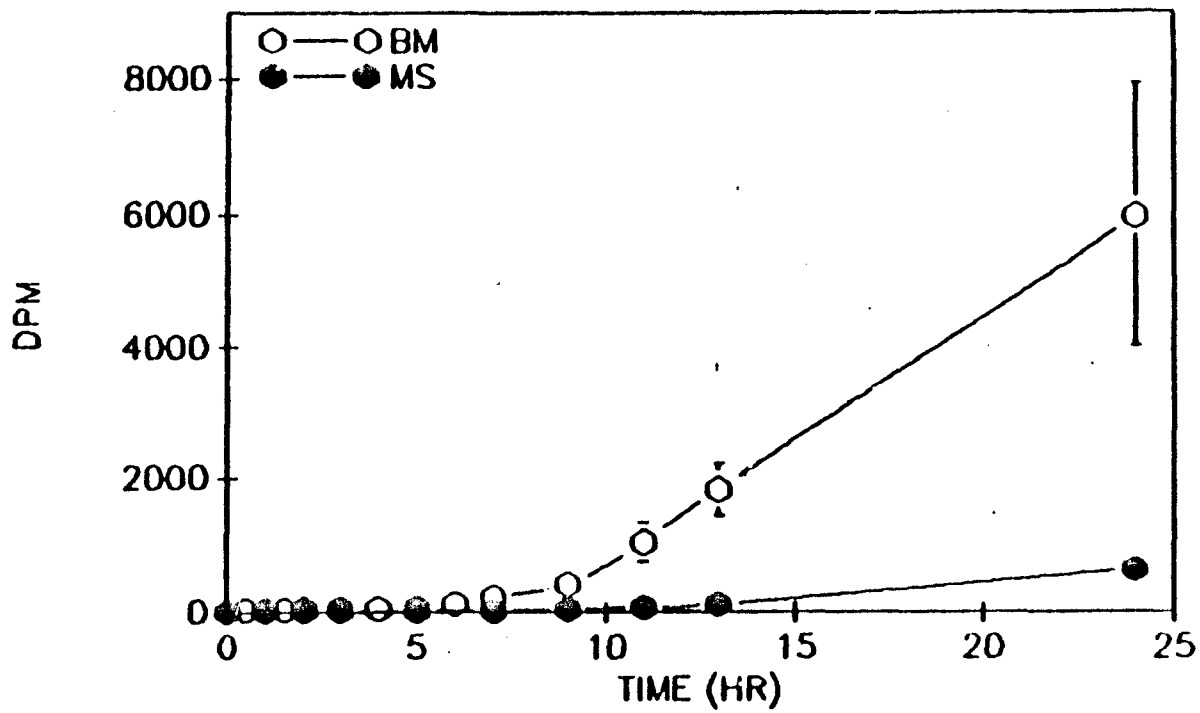


Figure 6A. Penetration of [3H]PbTx-3 through monkey buccal mucosa and monkey skin. BM = buccal mucosa, MS = monkey skin.

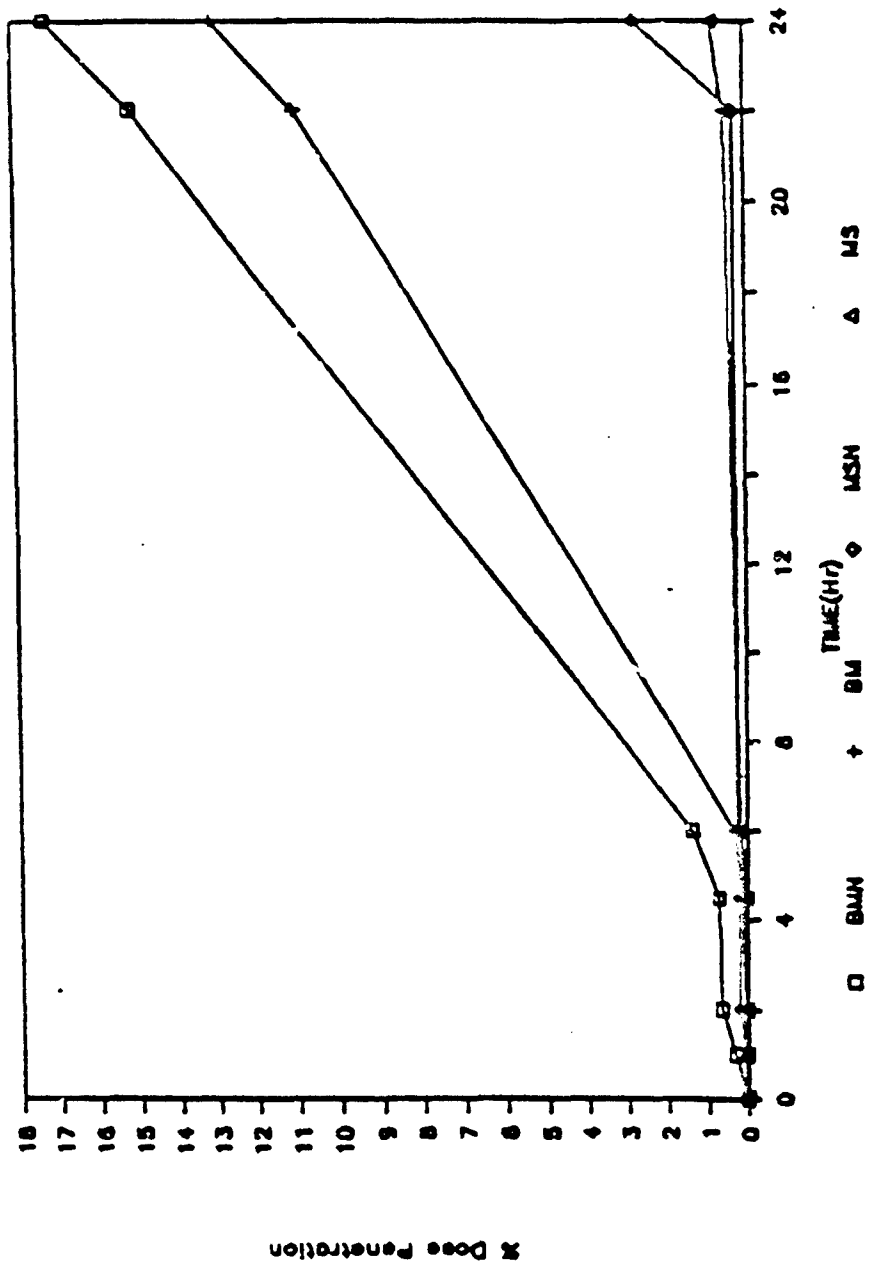


Figure 6B. Penetration of [3H]PbTx-3 through discs of excised monkey buccal mucosa and skin. BMN = buccal mucosa with nylon membranes, BM = buccal mucosa, MSN = monkey skin with nylon membranes and MS = monkey skin.

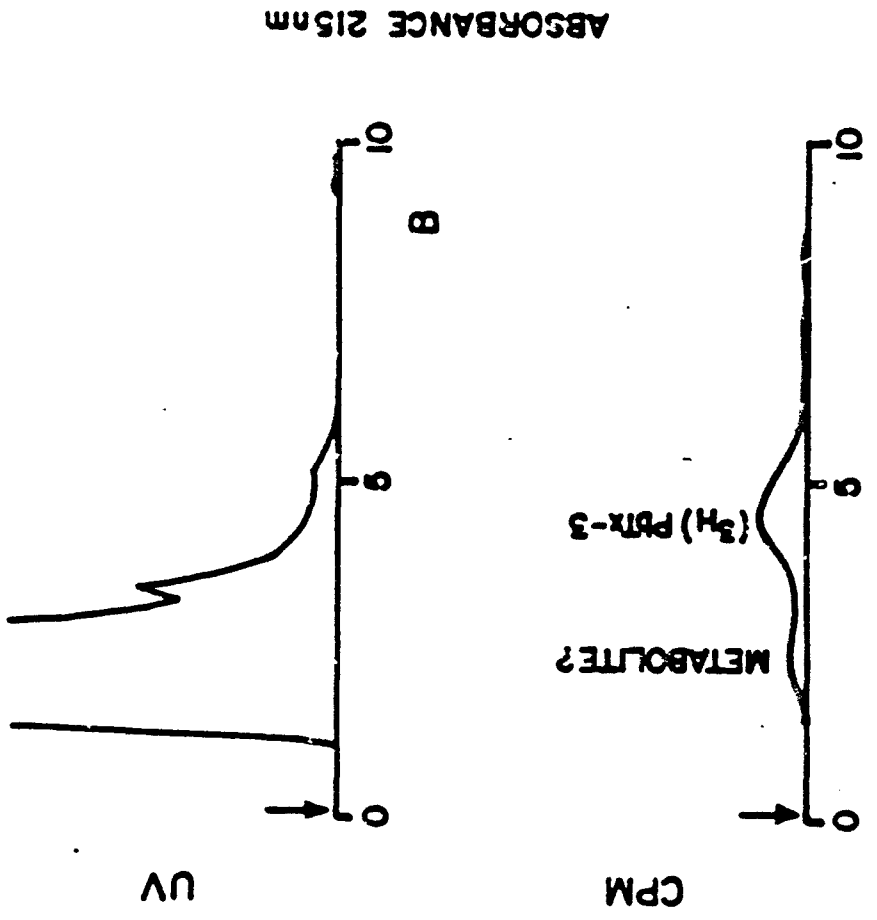


Figure 7. Chromatographic analysis of receptor fluid of tissue discs dosed with [3H]PbTx-3.

V. DEVELOPMENT OF EXTRACTION PROCEDURE AND CHROMATOGRAPHIC METHOD TO MEASURE LYNGBYATOXIN A IN SKIN EXTRACTS AND AQUEOUS RECEPTOR FLUID

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Barbara W. Kemppainen, Ph.D.
Meena Mehta, M.D.

STATEMENT OF PROBLEM

The purpose of this work was to determine (i) the amount of percutaneous absorption which would occur if humans were dermally exposed to low molecular weight (M.W.) toxins and (ii) the rate of penetration of low M.W. toxins through human skin and mucosal membranes. The toxin which is currently being studied is lyngbyatoxin A (teleocidin A).

BACKGROUND

In skin penetration studies of lyngbyatoxin the compound will be placed upon excised split thickness human skin mounted in a diffusion cell. The lower surface of the skin (dermal side) is bathed with Hank's Balanced Salt Solution (HBSS) into which the lyngbyatoxin will penetrate. This receptor fluid is aqueous in nature and not suitable for injection onto a normal phase chromatography column. Also the lyngbyatoxin is in extremely low concentration and must be concentrated prior to injection onto the HPLC. Therefore it is necessary to develop an efficient,

reproducible extraction method for lyngbyatoxin before proceeding with the skin penetration studies.

EXPERIMENTAL METHODS

1. High Performance Liquid Chromatography: A Waters Company HPLC system was used and consisted of a Model 510 pump, a U6K manual injector, a Model 740 integrator and a Model 490 programable wavelength detector. The column used was a Partisil 5 micron normal phase silica column from Whatman. The mobile phase consisted of hexane; chloroform; isopropanol, 85:10:5 % (v/v). The flow rate was 0.5 ml/min and the absorbance was monitored at a wavelength of 254 nm.

The purity of lyngbyatoxin A (teleocidin A) was determined with the HPLC. One vial of the lyngbyatoxin (0.05ug/ul) was determined to be between 93-95% pure with a peak of lyngbyatoxin A-2 (an isomer) coming out just after the lyngbyatoxin peak.

In chromatography it is desirable to have a linear relation ship between the detector response (absorbance) and the peak area as measured by the integrator. The linear relationship permits easier and more accurate quantitation of the amount of the substance of interest. In order to determine whether or not the detector response was linear, a series of four different amounts (in micrograms) of lyngbyatoxin A was injected. The results are seen in Figure 1. Following injection of between 0.0025 and 0.0175 micrograms the detector response was linear with a correlation coefficient of 0.9953. This work should be repeated for other amounts of lyngbyatoxin A and at other sensitivities.

2. Extraction Methods:

A. Solid Phase Extraction Columns: An effort was made to develop a sensitive and consistent method for measuring small amounts of lyngbyatoxin in 2.5 ml volumes of PBSA or HBSS (receptor fluids used in in vitro penetration experiments).The following method was used:

Spike 5 vials containing 2.5 ml PBSA with 1 ug of lyngbyatoxin A.

Prime normal phase SPE columns with one column of hexane (3 ml).

Apply sample to the column, aspirate.

Wash with one column hexane.

Collect analyte by eluting with 2 ml of chloroform.

Dry down chloroform eluate, reconstitute with methanol.

Inject aliquots on HPLC.

Results indicated that the mean (± S.D.) recovery of lyngbyatoxin A was 58.2 ± 16.5% (expressed as percent of amount spiked in 2.5 ml PBSA). Previous work obtained a recovery of 39 ± 34%.

The use of C_{18} and CN solid phase extraction columns were unfavorable. Even when using 10 ug lyngbyatoxin A in 1 ml of HBSS no lynbyatoxin could be recovered.

The decision was made to use a liquid/liquid extraction method to compare extraction efficiency and reproducibility with those results obtained using solid phase extraction columns (58.2%

± 16.5). Three organic solvents were selected for possible use in the liquid/liquid extraction method; chloroform, methylene chloride and hexane.

3. Liquid/Liquid Extraction: HBSS buffer (2.5 ml) was placed in a 5 ml conical bottom vial and 1 ug lyngbyatoxin A was added. The vial was shaken and 1 ml of HPLC grade chloroform was added. The vial was again shaken and then allowed to sit for 25 minutes. The chloroform was removed from the bottom of the vial using a pasteur pipet and placed in a 1 ml vial. The chloroform was evaporated under a stream of nitrogen and gentle heat. The sample was reconstituted with 200 ul of hexane and 10 ul was injected onto the HPLC. A standard was made from the same dilution of lingbyatoxin solution. Recovery of the lyngbyatoxin from HBSS was 26.7%.

A modification of the liquid/liquid extraction procedure was performed. HBSS buffer (2.5 ml) containing 1 ug lyngbyatoxin A was extracted three times with the organic solvent. The extracts were combined and evaporated under a stream of nitrogen and gentle heat. Using methylene chloride as the organic phase the recovery was 59.8%. Using hexane the recovery was only 1.72%. Chloroform was tried using the new procedure and the recovery was 69.2%.

During extractions and sample preparation for HPLC it is often beneficial to have an internal standard which can be used to calculate the recovery of the compound of interest such as lyngbyatoxin. A search for a suitable internal standard was initiated. One possible candidate is ethyl 3-indoleacetate. The compound was ordered from Aldrich Chemical Company. Dilutions of

ethyl 3-indoleacetate in chloroform were injected into the HPLC using the same conditions as for lyngbyatoxin. A peak was observed with a retention time between 5.4 and 6.3 minutes (see Figure 2b.), the peak area was proportional to the concentration.

It was necessary to check for naturally occurring skin components which might interfere by co-eluting with the internal standard and/or lyngbyatoxin. A piece of human cadaver abdominal skin was prepared with a dermatome. The skin was approximately 0.5 mm thick. A small disk of skin was placed on a static diffusion cell and the cell was filled with HBSS buffer to serve as receptor fluid. The diffusion cell had a water jacket which kept the cell at a temperature of 37°C. The receptor fluid was removed after 24 hours and subjected to liquid/liquid extraction with methylene chloride. The extract was evaporated, reconstituted with 200 ul hexane and injected onto the HPLC. The skin disk was placed in 2 ml of methylene chloride and sonicated for 10 minutes. The methylene chloride was removed and evaporated. The skin disk was extracted two more times and the extracts combined. The skin extracts were also reconstituted with 200 ul of hexane and injected onto the HPLC. The resulting chromatograms (see Figure 2.) showed that substances from the skin were present and eluted with retention times between 4.6 and 5.4 minutes. These substances interfered with the detection of the ethyl 3-indoleacetate peak but would not interfere with the lyngbyatoxin A peak. Spiking the extracts with ethyl 3-indole acetate further demonstrated interference. Increasing the proportion of isopropanol in the mobile phase from 5 to 13% reduced the retention times of the interfering

compounds but also decreased the retention time of the ethyl 3-indolaacetate.

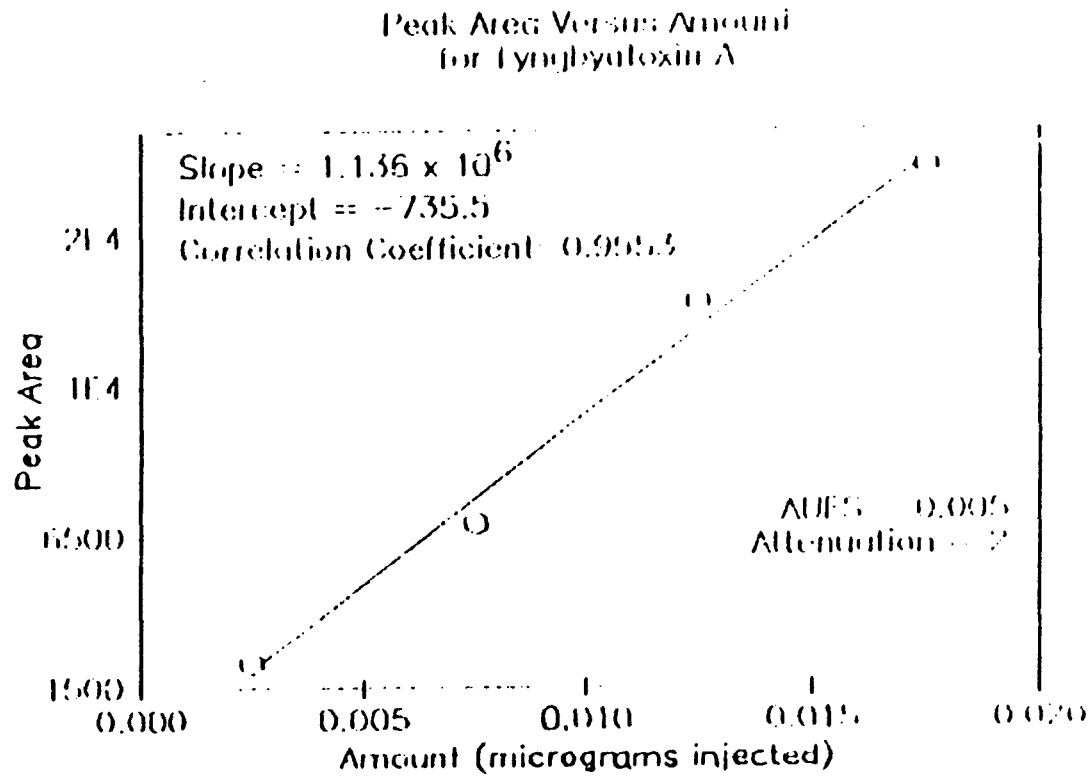

Figure 1. Graph of amount versus peak area for four different concentrations of lyngbyatoxin. This graph shows that the detector respose is linear with respect to concentration.

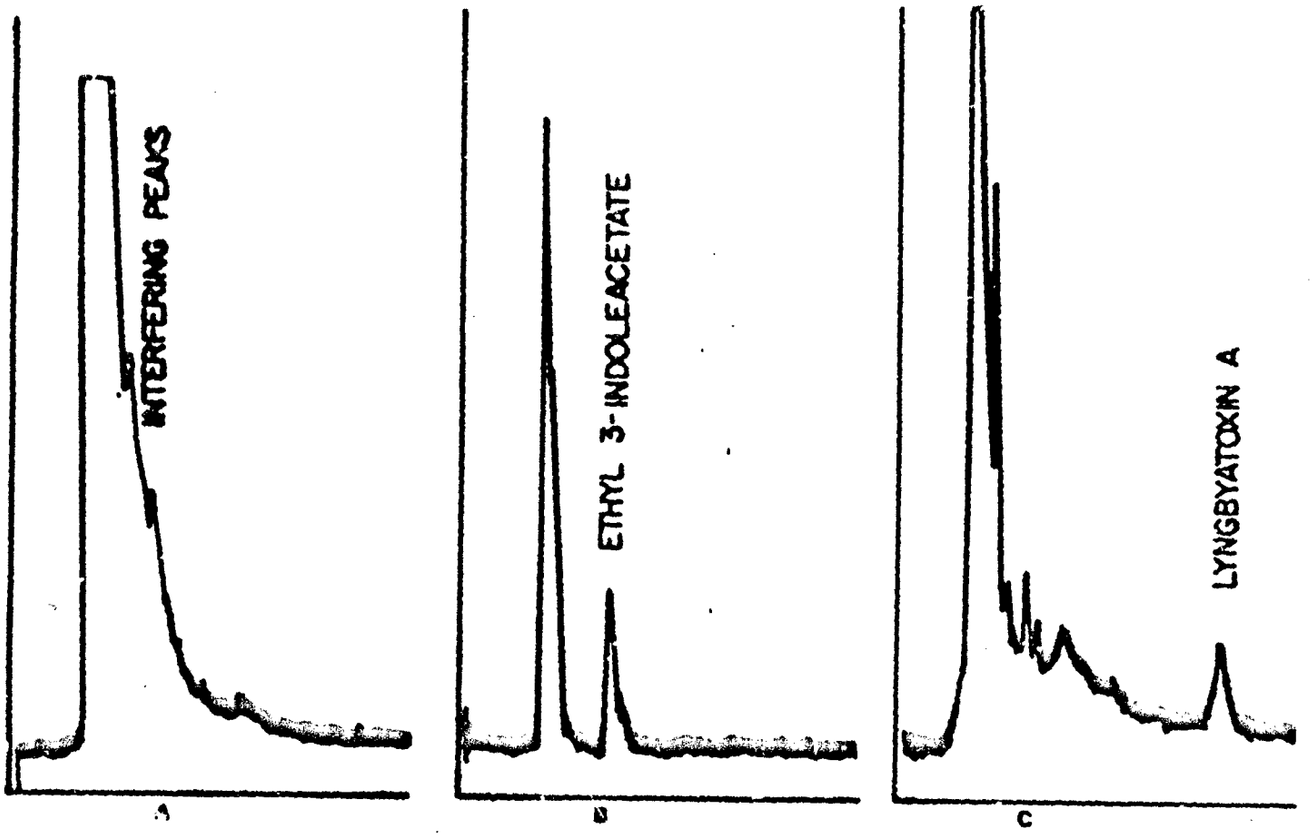

Figure 2. A) Chromatogram of Methylene Chloride Extract of HBSS receptor fluid bathing human skin. 10ul injection of extract evaporated and reconstituted with 200ul of hexane. B) Chromatogram of 5 ul injection of proposed internal standard ethyl 3-indoleacetate (0.0113ng/ul). wavelength = 254nm. C) Chromatogram of 1.0 ug of Teleocidin A extracted from 2.5 ml HBSS receptor fluid using a silica solid phase extraction column.

VI. EVALUATE VIABILITY OF EXCISED SKIN AND BUCCAL MUCOSA BY MEASUREMENT OF LACTATE DEHYDROGENASE AND HISTOLOGICAL EXAMINATION.

STATEMENT OF PROBLEM

Determine viability of excised skin and buccal mucosa by measuring the enzyme lactate dehydrogenase (LDH) and histological examination.

BACKGROUND

Lactate dehyrogenase is an enzyme found in cardiac, renal, hepatic, and muscle tissue. Erytrocytes also contain large amounts of LDH. Elevated serum levels are most commonly associated with myocardial infarction, renal infarction or trauma, acute hepatitis, disseminated malignancy and pernicious anemia. When cells die they release their high molecular weight cytosolic proteins into the extracellular fluid. One cytosolic protein is lactate dehydrogenase. The presence of this enzyme in extracellular fluid is an indicator of tissue damage. Because LDH is a large molecule it cannot easily penetrate the cell membrane. For this reason, high levels of LDH in extracellular fluids is an indicator of loss of viability. That is, release of LDH is equated with cell death.

LDH accelerates the oxidation of lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD) which is concurrently reduced to NADH. NADH has a high absorbance in the

ultraviolet region, peaking at 340 nm, whereas NAD shows essentially zero absorbance at that wavelength. Since the reduction of NAD proceeds at the same rate as oxidation of lactic acid, the reaction is followed spectrophotometrically by measuring the increase in absorbance at 340 nm caused by the formation of NADH.

EXPERIMENTAL METHODS

A.MEASUREMENT OF LEAKAGE OF CYTOLOGIC PROTEINS OUT OF CELLS

Hanks Balanced Salt Solution with HEPES buffer (HBSS) was bubbled with 95% O_2 and 5% CO_2. HBSS is used as receptor fluid in the skin penetration studies which we are conducting. HBSS has been reported to keep the skin discs viable (S.W. Collier, N.M. Shiekh et al, 1988). Full thickness excised guinea pig skin discs were placed in vials which contained 3 ml of HBSS (bubbled with 95%O2 :5%CO2) and were incubated at 36°C. This is the temperature at which skin penetration experiments were conducted. Sequential samples, each of 200 ul, were collected at time 0, 1 hr, 6 hr and 36 hr. Receptor fluid samples (10 ul each) were analyzed by spectrophotometer.

B. HISTOLOGICAL EXAMINATION

Human skin and monkey buccal mucosa was obtained for skin penetration studies. Loose subcutaneous tissue and fat was removed and tissue discs, 2.8 cm^2 in diameter were cut. For control, some of tissue discs were immediately fixed in formalin. Rest of the tissue discs were mounted on static teflon diffusion cells.

Receptor fluid used was HBSS bubbled with 95% O_2 and 5% CO_2. Cells were incubated at 36° C. Experimental conditions were similar to those used for penetration studies. Cells were taken apart after 1 hr, 2 hr, 6 hr, 12 hr, 24 hr, 36 hr and 48 hr. On taking the cells apart, tissue discs were fixed in formalin. Histopathological examination was done at ALABAMA Veterinary diagnostic laboratory (Auburn, AL).

RESULTS AND DISCUSSION

A. LDH DETERMINATION

Change in ultraviolet absorbance was plotted against time. Preliminary results show an increase in the amount of LDH with time. Maximum increase was observed during the first 2 hours, refer to figure 1. This increase in LDH could be due to damage caused to tissue during the process of skin preparation itself, that is, excising the skin and cutting the tissue discs.

B. HISTOLOGICAL EXAMINATION

Monkey buccal mucosa and human skin, incubated for 1,2,6 and 12 hr was found to be normal on histological examination. There were mild changes in the buccal mucosa and skin incubated for 24 hr and 48 hr, respectively. The mild changes which were observed included pyknosis and swelling of epithelial cells in the epidermis and hair follicles. Changes in the dermis included pyknosis of connective tissue cells, vascular degeneration and generalized swelling of the collagen bundles.

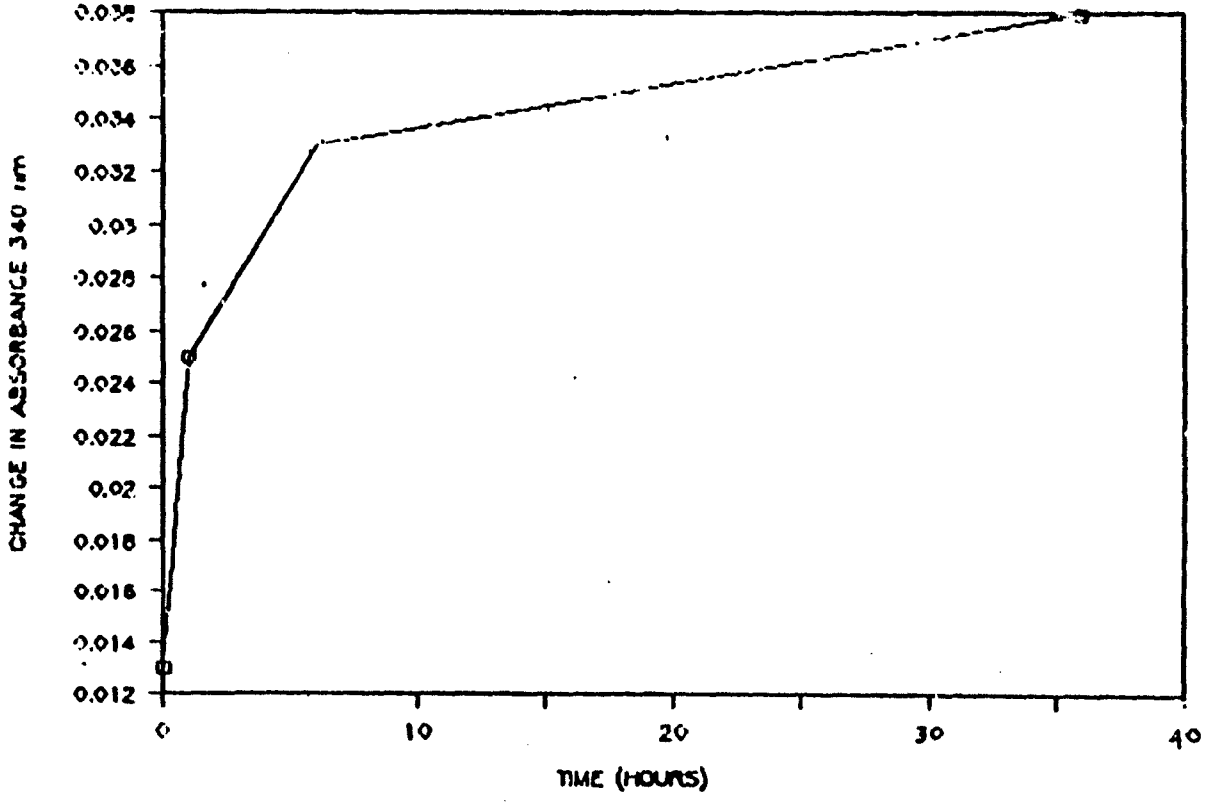

Figure 1. Preliminary measurement of lactate dehydrogenase in buffer bathing skin.

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